

The e-developing world

Participants in an international forum last month were appropriately fired up about the opportunities for the poorest countries that are provided by information technologies. The attitudes of governments can make all the difference.

Go up Hiei-zan, a mountain to the east of Kyoto, and you encounter a timeless, peaceful space: the vast Enryaku-ji temple complex, founded in the eighth century. Enter the most important building and you find, in the dimly lit and incense-laden atmosphere, statues of Buddha accompanied by flames kept burning for 1,200 years, with monks as guides and people kneeling in contemplation and prayer.

Look elsewhere in Kyoto and you will no doubt encounter a very different space: virtual rather than physical, and filled with continuously text-messaging teenagers who consider it impolite to take more than an hour to respond to a message on their mobile phones, even if they're in the middle of classes at school.

Participants at the Science and Technology in Society Forum, held last month in Kyoto (see www.stsforum.org), had a chance to see the former space and to discuss the development of the latter and other similar e-spaces. They heard about the growing ability of broadband mobile technologies to "put the world in your hand". They heard about the Grid, which takes gigantic computational or data tasks, breaks them down into streams of data packets, and distributes their processing around a vast computer network. This will give the fabric of the Internet the capacity not only to ingest terabytes of data in an hour or less, but also to compare the data with models, to seek patterns, and to test hypotheses in real time or even faster. This infrastructure is being developed with scientific and defence-related challenges very much in mind, but no doubt it will also contribute to the world in everybody's hand.

Except that it isn't everybody, of course. The people who stand to benefit most from these developments are the minority who live in the developed world. So how to enhance access for the rest? A discussion of "The e-developing world" focused on seeking solutions that were sustainable in a business sense. The participants included senior people from technology and telecommunications companies such as Sun Microsystems, Intel, Nokia and UTStarcom, as well as from universities and the US National Science Foundation. So, they were asked, is it realistic to expect that multinationals will target as a market the four billion people at the bottom of the human pyramid — the poorest on the planet — rather than just the billion at the top?

Spreading the word

Most of the participants were optimistic. After all, if a company has well established core markets, it can afford to go after other markets that build new revenues on marginal costs. There are already such companies, spurred on by business-positive governments in the emerging markets.

Two elements are essential. The first consists of imaginative ways of increasing the reach of the technology. The use of micro-enterprises is one example, such as the 'phone lady', a local village woman who sells minutes of Internet time. Governments can also play a role: for instance, the Kerala government in India is to provide some 7,000 villages with Internet cafés, each with ten computers, and will use them to deliver important services to the villages.

Another key element is micro-commerce. Information and communication technologies (ICT) can be used to reduce the cost of

credit, cut costs by removing the middle-man, and hook major banks reliably to micro-credit.

Success breeds success, so it is worth considering some inspiring examples. Text messages using 'short message service' — the same technology used by Kyoto's teenagers — are used to alert people to take tuberculosis medicine in South Africa. More sophisticated telemedicine in remote regions also has high potential payoff. The UN World Food Programme uses high frequency, very low bandwidth to communicate with trucks in its distribution network. The propagation of instructional materials has been effective — in Togo, West Africa, for example, Internet cafés are just a bicycle ride away. And one of the most successful examples of the use of ICT in developing regions has been 'price discovery' — using the technology to discover the price of commodities (such as soy beans) in large markets and then negotiating appropriately with the 'middle-man' buyer.

Political will

To speak of the 'developing world' is overly simplistic: there is a vast difference between small developing countries and China or India. And the difference between these giants is itself instructive, most fundamentally in the role played by government — at national, state and local levels. In China, these governments see themselves as a kind of competitive business, and work hard and proactively to bring about the kind of infrastructure they would like to see in their country, state, region or village. India, in practice, despite its success in information-technology service development, is seen as less welcoming territory for outsiders with technology to offer.

Educating open-minded government officials in best practice in ICT can have huge leverage. If governments see themselves as groups to be wooed by business, they will lose out. They must be proactive and work in partnership with other agencies and companies to increase the size of the cost-effective, reachable market for goods and services. Over the next ten years, the biggest heroes in the deployment of ICT in the developing world will not be the engineers or the business leaders, but the most effective political leaders.

Perhaps the most stimulating task given to the discussion by its chairman Richard Newton, dean of engineering at the University of California, Berkeley, was to come up with a prize challenge: what technological achievements for the developing world would merit a US\$10-million award? Three ideas stood out: an affordable computer-based tutor that could instruct an illiterate person to learn a useful activity within a month; a phone that translates between languages in real time; and a demonstrable reduction by at least two orders of magnitude in the cost per bit of information delivery.

Whatever politicians and businesses might achieve, technologists should not lose sight of the need for simplicity. This is essential for illiterate people in remote villages in the poorest countries. It is also important for older people everywhere who want nothing to do with the Internet and yet could benefit so much from communicating more readily with their grandchildren.

For those who live in the e-developed world, the challenge is increasingly becoming how to switch off. Those lucky enough to visit Kyoto should start by visiting the temples on Hiei-zan. ■





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Suppressed study raises spectre of flawed drug regulation in US

Emma Marris, Washington

Amid claims that it suppressed publication of a study into the safety of a painkiller, the US Food and Drug Administration (FDA) is under increasing pressure to reform the way in which it monitors approved drugs.

Vioxx, a prescription painkiller made by New Jersey-based Merck, was withdrawn by the company on 30 September after a study it had commissioned linked the drug to an increased risk of heart attacks.

But the FDA — the world's largest drug regulator — is facing detailed allegations that it pressed one of its top drug-safety officials to withdraw a paper on Vioxx from publication in *The Lancet*. The study, led by David Graham, associate director for science at the FDA's Office of Drug Safety, also linked the drug to heart attacks. Graham estimates that Vioxx has been responsible for several thousand deaths since it was approved in 1999.

On 18 November, Graham, a 20-year FDA veteran, vaulted into the national spotlight when he testified at a Senate hearing on Vioxx that the agency was "broken". The hearing raised questions about why the FDA waited for Merck to take action, when Graham's preliminary data earlier in the year had suggested that the drug should be withdrawn.

Now the agency is under attack for suppressing Graham's Vioxx paper, which he hoped to publish at the time of the hearing. According to extracts from e-mails printed in *USA Today* on 29 November, Steven Galson, acting director of the FDA's Center for Drug Evaluation and Research, contacted editors at *The Lancet* and made reference to an internal FDA report that contained allegations that Graham might have manipulated data in his study.

Richard Horton, *The Lancet's* editor, reacted with irritation. "One could read such an allegation as an attempt to introduce doubt into our minds about the honesty of the authors," he wrote, "doubt that might be sufficient to delay or stop publication of research that was clearly of serious public interest."

The FDA said in a statement that Graham had submitted the paper "without going through the long-established peer review and clearance process established for scien-



Safety check: David Graham testifies at a Senate hearing, claiming that the FDA is 'broken'.

tific papers submitted by FDA scientists".

Graham says that the charges of data manipulation arose from corrections that he made between an earlier abstract and the final version of the paper. He ultimately withdrew the paper on 16 November, saying that he feared for his job. "I got a very explicit e-mail from Dr Galson saying I could not let it be published, and if I did, I and *The Lancet* would be responsible for the consequences." He says he may now remove his name from the study so that it can be published without the need for FDA approval. "The FDA is engaged in an act of scientific censorship," he claims.

Under pressure

Graham says he is now being pressured by Lester Crawford, the FDA's acting commissioner, to accept a transfer to a desk job, and expects to be ordered to move within the week. "I am a scientist," he says. "Removing me would be an act of retaliation."

In a statement, the FDA said that it does not "condone any form of employee retaliation. In fact, as we have repeatedly stated, we encourage internal scientific debate."

Alastair Wood, an associate dean of Vanderbilt University in Nashville, Tennessee, and

one-time contender for the post of FDA commissioner, slammed the agency's response to the Vioxx affair, which he says probably killed more people than the 11 September terrorist attacks. "We've had a major public-health disaster," he says. "Yet we have no forum for open discussion, no way to move forward. Instead, each side has got into their bunkers."

And Vera Sharav, president of the Alliance for Human Research Protection, a New York-based patient-advocacy group, thinks the whole agency badly needs shaking up. "The leadership has to go," she says. "A firewall has to go up between FDA reviewers and industry."

But criticism of the drug-safety agency is reaching far beyond vocal groups such as Sharav's. *The Journal of the American Medical Association* has just published an editorial calling for the Office of Drug Safety to be "decoupled" from the rest of the FDA (P. B. Fontanarosa *et al.* *J. Am. Med. Assoc.* **292**, 2647–2650; 2004). And Senator Chuck Grassley (Republican, Iowa), chair of the Senate finance committee, says that he will introduce legislation to move it from "under the thumb" of the Office of New Drugs, the powerful branch of the FDA responsible for drug approval.

Europe breaks ranks over fusion project site

Declan Butler, Paris

The multibillion-dollar ITER fusion reactor was always intended to be an international collaboration, and was billed as a model for 'bigscience' projects. But this week, the model looks in deep trouble.

On 26 November, the European Union (EU) broke ranks with the site selection process, and announced that if agreement isn't reached quickly, it will go it alone to build the reactor in Europe.

ITER aims to prove the principle of generating energy from fusion, by confining a ring of plasma with a magnetic field in a doughnut-shaped vessel and heating it to several million degrees kelvin. But the six-strong collaboration has split over the siting of the facility: China and Russia back the EU's site in Cadarache, France, whereas the United States and South Korea favour Japan's rival site at Rokkasho.

At a meeting in Brussels, research and industry ministers from the EU's 25 member states followed through on a threat that they first aired in September. They authorized the European Commission, the EU's executive arm, to abandon the multilateral site selection process if necessary and build ITER in France, with as many outside partners as it can muster.

EU ministers insist that this option will be a last resort. The Japanese government has yet to respond, but fusion officials there reacted angrily. Satoru Ohtake, director of fusion energy at Japan's science ministry, says the EU move jeopardizes not just ITER



Site fight: Europe is holding out to build ITER at Cadarache in France.

but also the ground rules needed for future international collaborations. "By pursuing their own desires, they will be the ones that break international trust," says Ohtake. "This is divisive; it is not acceptable to us."

Commission officials counter that the stalled talks needed shock therapy and that the situation was "in complete deadlock". Political realities make site selection the inevitable crux of any international collaboration, adds one official, and "unless your project is in space, you will have always this problem of choosing the site".

Europe's most recent offer to Japan, in return for agreeing to the Cadarache site, includes a fusion package that would compensate the country and include support for a major upgrade to its JT60 fusion reactor. Ohtake claims that Japan has offered Europe similar concessions.

Neither offer prevailed, however, at the

latest meeting of negotiators on 9 November in Vienna (see *Nature* 432, 262; 2004). Japan's position is that both parties should temporarily put aside the siting issue, says Ohtake, and discuss the "role of host and non-host parties".

Europe's brinkmanship seems partly based on the reckoning that only one ITER will ever be built, and if it can force the deadlock with firm support for a French site, then Japan and the United States will eventually join the only show in town.

This scenario is credible, says Stephen Dean, head of Fusion Power Associates, a Washington-

based fusion advocacy group. "I think the EU could get together a package that would work," he says.

France has already offered to double its support for ITER to \$1.12 billion, or 20% of the total bill, and the rest of Europe has committed another 40%. Even if Japan and the United States pulled out, Europe could conceivably make up the shortfall from Russia, China and other partners.

But some European fusion scientists fear that the EU has overplayed its hand — and underestimated Japanese determination to host ITER. "The European Commission badly misread Japan's negotiating positions; it has been convinced that Japan would budge," says one European scientist close to the negotiations. The big unknown, he says, is whether Japan will call Europe's bluff, and announce that it, too, intends to go it alone. ■

Additional reporting by Geoff Brumfiel in Washington.

Scientists decry 'spy' verdict for Russian physicist

Bryon MacWilliams, Moscow

A Russian physicist has been sentenced to 14 years in prison after a retrial found him guilty of passing classified information to China. The verdict has been derided by scientists and human-rights observers.

Valentin Danilov admitted selling aerospace technology to a Chinese company in 1999, when he was head of the Institute of Thermodynamics at Krasnoyarsk State Technical University in Siberia.

In 2001, he was arrested by the Federal Security Service (FSB), who said his device to measure the effects of electromagnetic waves on satellites could help China to develop weapons that might threaten Russia.

But Danilov, with the public backing of prominent members of the Russian Academy of Sciences, countered that the

technology was declassified in 1992 and that details of it had, in fact, been published in scientific journals. At least one of these publications is still openly available today.

The FSB has accused several scientists and environmentalists of espionage in recent years — something that human-rights groups refer to as "spy mania". But last year, in a startling setback for the agency, a jury sided with Danilov and acquitted him of all charges.

Prosecutors successfully appealed the verdict on procedural grounds and won a second trial. During this one, jurors were permitted to decide only whether Danilov had provided the information, and not whether it constituted spying. That decision was left to Judge Andrei Afanasyev of the Krasnoyarsk Regional Court, who

ruled that the data were indeed secret.

The 24 November verdict was questioned by Eduard Kruglyakov, deputy head of the Budker Institute of Nuclear Physics in Novosibirsk, who said that the judge had refused to consider written testimony from Russian physicists in relevant subdisciplines.

Afanasyev also found Danilov guilty of embezzling 466,000 rubles (\$16,500) — the first payment by the Chinese company. During the retrial he unexpectedly jailed Danilov, who had been free, after prosecutors accused the physicist of "criminal activity" for giving an interview to a US newspaper, the *Los Angeles Times*.

Danilov's lawyer, Yelena Yevmenova, says she will appeal the sentence in the Russian Supreme Court, as well as the European Court of Human Rights. ■



High stakes: conservationists fear that overfishing may destroy the bluefin tuna population.

Plans to track tuna canned amid claims of cash shortfall

Rex Dalton, New Orleans

A research programme to investigate populations of Atlantic bluefin tuna has been shelved by the international commission charged with conserving the fish.

The first phase of the project failed to win approval at the meeting of the International Commission for the Conservation of Atlantic Tunas (ICCAT) in New Orleans, which ended on 21 November.

ICCAT officials say that its €2-million (US\$2.6-million) annual budget cannot accommodate the €250,000 initial cost of the research programme. But scientists and environmental groups who back the programme claim it was scuppered because some ICCAT parties don't want to hear the likely results of the research.

In particular, they charge, European government officials are worried that if stronger links are established between the bluefin populations on the two sides of the Atlantic Ocean, there would be greater pressure on them to cut European tuna fishing.

Some say that ICCAT's failure to pursue the research will hasten the collapse of the bluefin population. "It's a nightmare," says marine biologist Paolo Guglielmi, who heads the Mediterranean fisheries programme for environmental group the WWF in Rome. "Everybody sees the danger approaching, but no one can agree on how to escape it."

"What the commission is doing is very short-sighted," agrees John Graves, a fisheries geneticist at the College of William and Mary in Virginia, who chaired a committee of advisers to the US delegation to the meeting. "Ideally, the management of the fishery should be based on science. That is not happening."

European Union (EU) representatives at the meeting argued that ICCAT couldn't afford the research project. John Spencer, head of the EU delegation, declined to be interviewed for this article. In contrast, US officials argued unsuccessfully in favour of the programme.

ICCAT currently allows an annual quota for the eastern Atlantic of 32,000 tonnes of bluefin, but even its officials acknowledge that the actual catch is probably more than 40,000 tonnes. Fishermen in the Mediterranean say that they would probably benefit from more research and tighter quotas on their catches. But Graves claims that EU officials don't care what happens "because when the bluefin population plummets, they will already be gone".

The research proposal was developed in May 2003 by an international panel appointed by ICCAT. It would aim to develop a better understanding of the bluefin's life cycle, and to determine if there really are two separate stocks — one in the east spawning in the Mediterranean, and one in the west Atlantic spawning in the Gulf of Mexico.

The fish are currently managed as two separate stocks, and fishing in the west Atlantic is far more tightly restricted, with an annual quota of just 2,700 tonnes. But studies of electronically tagged bluefin indicate that some fish cross the ocean, and many marine biologists think that the population in American waters is being depleted by European fishing.

Although the scientists were unsuccessful in New Orleans, Masanori Miyahara, a Japanese official and chairman of ICCAT, said he was optimistic that a research programme might be approved when the commission holds its next meeting in Japan next April. ■

Nuclear agreement paves way for fuel recycling in Japan

Ichiko Fuyuno, Tokyo

Japan's bid to recycle nuclear fuel at home came closer to reality this month, with the announcement that a massive, US\$27-billion recycling plant is set to begin trial runs early next year.

On 22 November, local government officials in northern Aomori, the prefecture where the plant is being built, said that they had signed a safety agreement with its operator, Japan Nuclear Fuel Limited, to allow the tests to go ahead.

The decision had been eagerly awaited by Japanese power companies, who see recycling as essential for the long-term future of the country's nuclear-power industry. Japan currently gets about one-third of its electricity from nuclear power.

Aomori had hesitated to approve the tests because of safety problems involving faulty welds and leaks of radioactive water from the plant's spent-fuel storage pool.

But earlier in November, Japan's Atomic Energy Commission endorsed the recycling plan, and Aomori's licensing decision opens the way for the plant to begin operating. The prefecture's decision is "remarkably important", says Yohsaku Fuji, chairman of the Federation of Electric Power Companies of Japan.

The trial runs will last for a year and use depleted uranium, a by-product of nuclear-fuel processing in Japan, instead of spent nuclear fuel. If it is successful, the plant could start reprocessing spent fuel in 2006.

Japanese power companies plan to burn the reprocessed fuel, which contains plutonium as well as uranium, in their existing nuclear-power stations. The country eventually hopes to use the fuel in 'fast-breeder' reactors, although their development has been stalled since an accident at a prototype reactor in 1995.

Japan's reprocessing plans have been heavily criticized for their high costs — and because they produce material that could theoretically be used in nuclear weapons.

In addition, the plans do not address how Japan should dispose of an estimated 200 tonnes of spent nuclear fuel a year that the recycling plant can't accommodate. Japan has said this will be addressed in 2010. "The government has put off the disposal problem, which would affect Japan's entire nuclear-power policies," says Hajimu Yamana, a nuclear-energy professor at Kyoto University. ■

Croats protest that science minister is 'meddling' in MedILS

Federica Castellani, Munich

Croatian scientists are up in arms over possible government interference in plans for a world-class research institute on the Adriatic coast.

The Mediterranean Institute for Life Sciences (MedILS) would be established in Split, in the disused summer home of ex-Yugoslav leader Marshal Tito. The institute is the brainchild of Miroslav Radman, a Croatian biologist now at Necker Medical School in Paris and scientific adviser to Ivo Sanader, the Croatian prime minister.

But Croatia's research community has become increasingly concerned that Dragan Primorac, the science minister, is trying to stack the institute's advisory board with faculty members from the University of Split. Primorac founded the university's laboratory for clinical and forensic genetics in 1994 and ran it for nine years.

Radman has been threatening to withdraw from the institute in protest at what he sees as Primorac's meddling. "It was fundamental to the concept of MedILS that it should be under the leadership of an international scientific board, totally independent of local governments," he says.

And more than 1,000 Croatian researchers around the world have signed an Internet petition supporting Radman.

The project was endorsed by Ivica Racan, former prime minister of Croatia, in March 2003, but its future has been in doubt since he lost power to Sanader eight months later.

On 20 November, the Croatian government sought to end the uncertainty with the publication of a tender to complete the refurbishment of the building by next May. The government will pay for the renovation of the building and offer its use, rent-free, for 40 years. Radman will seek funds to support research and education there.

MedILS could boost Croatian research, says Kresimir Pavelic, head of molecular medicine at the Rudjer Boskovic Institute in Zagreb. "We are very provincial in our scientific outlook, and we need to grow internationally," he says. "That's why something like MedILS is so important to us."



Applause for thought: Ujjal Dosanjh has promised compensation for Canadian hepatitis sufferers.

Canada pledges cash for more victims of blood-bank debacle

David Spurgeon, Montreal

The Canadian government has promised to broaden its compensation package for victims of a blood-transfusion scandal that hit the country in the 1990s. The change means that an extra 6,000 people who contracted hepatitis C from Canada's national blood system should soon be eligible for financial awards.

"Compensation, quite frankly, should have been paid a long time ago," says Conservative opposition health critic Steven Fletcher. "I think the government has just given up trying to defend an indefensible position."

Problems with blood banks became apparent in several countries in the 1990s, with the realization that stocks had not been properly screened for viruses such as HIV and hepatitis, owing to a lack of knowledge and inadequate tests.

In 1998, when Jean Chrétien was prime minister, the Canadian government decided to compensate people who contracted hepatitis C from the national blood system — but only if they were infected between 1986 and 1990, when it said adequate tests were available but not used. This was despite an independent inquiry in 1997 that recommended compensation for everyone harmed by infected blood (see *Nature* 390, 432; 1997). The package, offered on condition that recipients would not sue, was for about Can\$25,000 (US\$21,000) per person.

Some provinces made money available for the 'forgotten victims' who were infected before 1986 or after 1990. But groups such as the Canadian Hemophilia Society campaigned for extended federal compensation, and some victims launched class-action suits.

The current Liberal government, elected in June, has now promised to open negotiations for those left out of the original Can\$1.1-billion compensation scheme. It says the new package will be sorted out with lawyers over the next few months.

Health minister Ujjal Dosanjh, who made the announcement last week, says the reason for the offer is that circumstances have changed. Some 10,000 people have so far applied for compensation — far fewer than the original government estimate of 22,000 — leaving about Can\$869 million of the original fund unclaimed. Treatment for hepatitis C has also improved, says Dosanjh.

But observers say there are more political reasons for the decision. Canada's current prime minister, Paul Martin, is a bitter rival of Chrétien, and the new package acknowledges implicitly that Chrétien's government was wrong to limit compensation. Reform of the national healthcare system was a major plank in Martin's election campaign.

The Canadian Hemophilia Society welcomes the announcement by Dosanjh, but adds that the payments have not been been adjusted for inflation, and that a large number of those affected have already died.

Some 80% of those infected with hepatitis C initially show no symptoms. Infection eventually becomes chronic in 75–85% of cases, often causing liver disease and sometimes proving fatal. Victims' groups say that many are now dying or struggling financially because they cannot work and the drugs they need are not covered by medical plans. Drugs that bolster the immune system are effective in 50–75% of cases, depending on the type of infection.

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Geneticists prepare for deluge of mutant mice

Alison Abbott, Bar Harbor

Off-the-shelf, made-to-measure mouse models of any human disease could be available sooner than you think. Medical researchers will be able to select their perfect model from a catalogue of mutants covering every single gene, specialists say.

Researchers who run the world's biggest mouse archives met in Bar Harbor, Maine, on 19–21 November to discuss how to handle the 300,000 or so new lines of mice that could be generated over the next two decades, expanding their stocks by almost a hundredfold.

"We will be hit by the start of the deluge within five years," predicts Steve Brown, head of the Mouse Genome Centre in Harwell, UK. International consortia of mouse geneticists have already announced plans to launch the first large-scale programmes to generate mutants systematically, gene by gene (see 'It's a knockout', below).

About a dozen 'mouse resource centres' were represented at the meeting, including the Rome-based European Mouse Mutant Archive (EMMA), the Jackson Laboratory in Bar Harbor and the RIKEN BioResource Center in Tsukuba, Japan. The centres collect the mutant mice made by scientists and distribute them to other researchers on request.

At the meeting, they decided to form an alliance that will share information through a single website, avoid duplication of lines, and present a single global voice when pressing for more funding. "We have to move from being a cottage industry and open ourselves globally to an international biomedical community," says Brown.

The glut of new mouse strains is expected to follow completion of the human and mouse genomes. The best way to make sense of this information is to see what happens to a living organism when individual genes are manipulated. Mice have proven particularly easy to alter genetically, so have become the most-wanted lab species.

The mouse is reckoned to have between 22,000 and 25,000 genes, and biologists would like to have a choice of perhaps ten or more types of mutant per gene. New cryogenic technologies that allow the freezing of viable mouse sperm or embryos created by *in vitro* fertilization will help ensure the archives are not overburdened with live mice.

"We don't want too many cages with live animals," says EMMA chief Martin Hrabé de Angelis. There are other advantages of not keeping live mice, he says. It has become very hard to ship live animals, particularly across borders. And there is the problem of 'gene drift' — the natural accumulation over generations of DNA variation as a result of background mutation. Another difficulty is that the characteristics making a particular mutant interesting can disappear.



Pooled resources: a global network will soon make genetically modified mice readily available.

But for the moment, few labs can handle frozen embryos or sperm, so specialist repositories still do most of the 'reanimation', making the mice available as breeding pairs within three or four months. Archivists at the meeting noted that to exploit mouse mutant resources, many more labs should learn how to reanimate animals themselves.

Many medical researchers are not even aware that the mutant archives exist and can offer them useful models — while those who do know about them often fail to offer the archives new mutants they have created. The archivists pledged to organize themselves into a global network committed to educating researchers and sharing resources. ■

It's a knockout

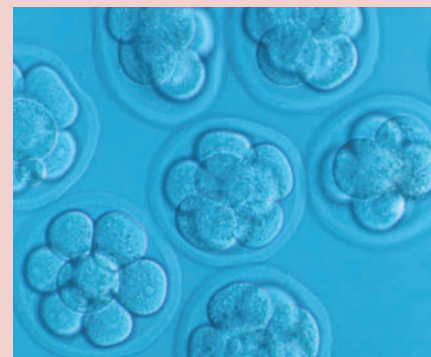
Geneticists around the world are pushing ahead with plans to scale up production of new mouse mutants for use as models of human disease (C. P. Austin *et al. Nature Genet.* **36**, 921–924, 2004 and J. Auwerx *et al. Nature Genet.* **36**, 925–927, 2004).

A US-based consortium is being spearheaded by Francis Collins, director of the National Human Genome Research Institute in Bethesda, Maryland, and former head of the international Human Genome Project. Its approach involves systematically knocking out mouse genes one by one in embryonic stem cells, which can later be used to make embryos.

This simple knockout technology is fast and cheap, but it has two disadvantages. Up to 15% of genes are needed for embryonic development, so any stem cell missing one of these will not develop into a mouse. And some knockout mice find ways to compensate for the lost function of the knocked-out gene and so appear normal.

Nevertheless, Collins estimates that stem cells covering most of the mouse genome will have been created within five years. He says that he is about to begin a worldwide e-mail survey to find out which knockout mice already exist, and to catalogue those available to researchers. He will then organize a workshop to discuss the implementation of the project.

Early next year, the US National Institutes of Health will call for proposals from scientists



Making mutants: scientists hope that genes vital for embryos could be switched off later.

interested in participating. "Money will be tight — I've just seen my budget figures — but the scientific need is compelling," Collins says.

A European-based consortium will focus on knocking out embryonic stem cells containing genes that the experimenter will be able to switch on or off at will in the mutant mouse. A gene required for embryo development, for example, could be switched off only after birth, or a gene associated with a disease of old age could be switched on only in ageing mice. But this approach will be much more time-consuming and expensive than the US strategy. The consortium is nonetheless seeking support from the European Commission and hopes to start work next year.

M. FRAY; FROZEN EMBRYO AND SPERM ARCHIVE, MRC-HARWELL

FROZEN EMBRYO AND SPERM ARCHIVE

Swiss voters give emphatic thumbs-up to stem-cell research

Paris Voters in Switzerland have backed legislation allowing stem-cell research in a referendum on 28 November.

Around two-thirds of those who turned out voted in favour of the law, which allows researchers to extract stem cells from human embryos left over from fertility treatment. The law was originally passed by the Swiss parliament in December 2003, but opponents, including the Green party, anti-abortion groups and critics of genetic engineering, subsequently collected enough signatures to put it to a referendum.

The vote makes Switzerland's rules roughly equivalent to those in France, the Netherlands, Denmark and Spain.

Ban on shark-finning wins transatlantic approval

New Orleans An intergovernmental fisheries group has agreed to ban the killing of Atlantic sharks for their fins. The International Commission for the Conservation of Atlantic Tunas (ICCAT), which represents 39 countries, approved the plan on 21 November — it will also



For the chop: shark carcasses are often discarded when the prized fins have been removed.

require fisheries to collect data on hard-hit shark species.

The widely criticized practice of slicing off shark fins and discarding the carcasses has already been prohibited by some countries, including the United States, as shark populations have been damaged by the practice. Fins are taken predominantly for use in soups, which sell for up to US\$100 a bowl. The fins have little taste, but are regarded in traditional Chinese medicine as a tonic.

Fishing vessels will be required to report all shark catches, including accidental by-catch, providing important data for research on declining populations, says Sonja Fordham, an analyst with The Ocean Conservancy, based in Washington.

"We are hopeful that regional fishing

organizations who govern other waters will follow ICCAT's lead," says Fordham.

Congress reins in Bush plans on nuclear arms

Washington Congress has eliminated funding for research on new nuclear weapons, along with money for several facilities that would have produced nuclear components.

When Congress passed the 2005 budget on 20 November, it removed \$27 million requested by the Bush administration for research into a new kind of nuclear bomb that would strike at deeply buried targets. The administration claims that the weapon, built by modifying existing nuclear devices, would be an effective deterrent. But outside scientists claim it would more probably be ineffective and dangerous to use (see *Nature* 428, 892; 2004).

Also removed from the bill was \$7 million earmarked for a facility to build the plutonium parts that act as nuclear triggers for most warheads. And \$9 million for research into advanced weapons concepts has been redirected into ongoing programmes to maintain current warheads.

Congressman Edward Markey (Democrat, Massachusetts) says that eliminating the programmes is the greatest victory for arms-control advocates in



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Congress for more than a decade. "This should send a strong signal to the Bush administration to drop this weapon from next year's budget request," he says.

Cash settlement provides sweet taste of success

Tokyo A researcher has pocketed ¥150 million (US\$1.5 million) in an out-of-court settlement with the Japanese seasoning company Ajinomoto for his work on the artificial sweetener aspartame.

The researcher, Masayoshi Naruse, developed a production method for aspartame with colleagues in 1982. Ajinomoto subsequently made almost ¥8 billion from the product, and Naruse originally asked for ¥2 billion. The amount he won was much less than this, but was still a "historic" sum, says Naruse's lawyer.

The settlement follows a similar one earlier this year, when researcher Shuji Nakamura was awarded ¥20 billion for his work on blue LEDs (see *Nature* 427, 478; 2004). Traditionally, Japanese scientists have been satisfied with little reward because they thought of themselves as part of a corporate family, says Masabumi Suzuki, a law professor at Nagoya University in Japan. These cases could encourage them to seek better financial treatment, he adds.

Chemistry cuts provoke heated reaction

London Britain's University of Exeter is threatening to shut down several departments in response to budget cuts, including chemistry, Italian and music, prompting protests from students and Nobel laureate Harry Kroto.

Irate students hit back by putting the university campus up for sale on the Internet auction site eBay on Wednesday 24 November — bidding reached £31,437 before the spoof offer was taken down by site officials on Thursday. Hundreds of students then went on a protest march. And Kroto, who won his Nobel prize for discovering 'buckyballs' — tiny balls of carbon that have been useful in nanotechnology — says he will return his honorary degree from the university in protest.

The university says that the changes are needed because of funding cuts, despite a 21%



rise in applications to study chemistry in 2004.

Exeter officials say that staff will be offered short-term contracts to continue teaching the threatened courses until the end of the year. The university's governing body will meet to discuss the closures on 20 December.

Transgenic-crop report provides easy weeding

London Using transgenic crops can make it cheaper to keep weeds under control, without negatively affecting biodiversity, according to a study released this week.

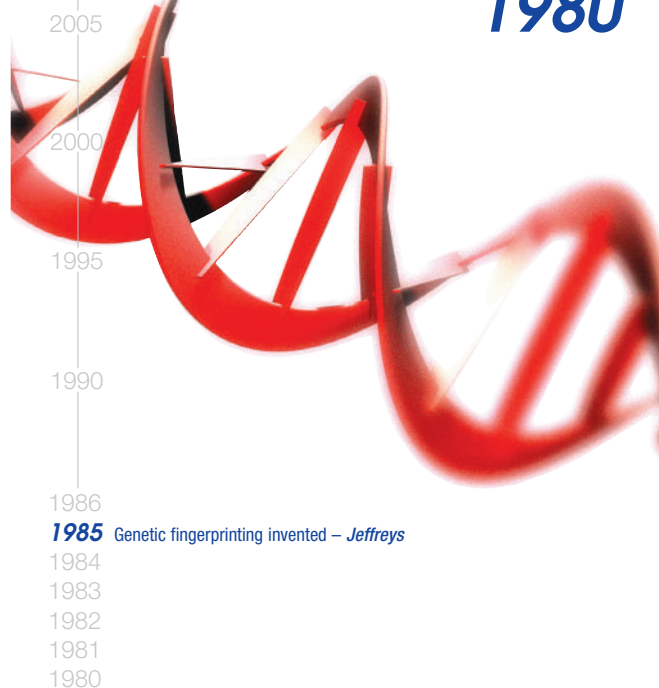
The Botanical and Rotational Implications of Genetically Modified Herbicide Tolerance study — conducted by a consortium of public and private partners and sponsored by the UK government —

looked at the effects of alternating between transgenic sugar beet or oilseed rape and conventional wheat or barley.

The number of weed seeds — a measure of biodiversity — increased by about the same extent on transgenic and conventional plots. But weedkillers for conventional rape cost £60 (US\$110) per hectare, compared with £16 and £40 for two transgenic strains.

The herbicide regime, rather than the crops themselves, has the greatest impact on the environment, the researchers say.

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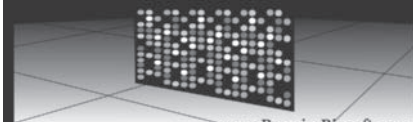
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Dispatches from the front line

Bad news travels fastest. Or so the scientists fighting disease hope. Since 1994, ProMED-mail has been reporting outbreaks as soon as they happen. Erika Check meets the team behind the 24-hour service.

Eleven years ago, a small group of scientists met in Geneva, Switzerland, to try to solve a life-or-death problem. They were facing up to a frightening fact: in a race against any outbreak of disease, they would lose. Tiny incidents in remote corners of the world could spread across continents in days or weeks — much faster than the doctors themselves could spread the word about a deadly scourge. The scientists needed a way to communicate quickly, to solicit advice about strange new infections, and to warn colleagues elsewhere to anticipate trouble. They placed a bet on a new, little-known technology: e-mail. And so the ProMED system — the Program for Monitoring Emerging Diseases — was born.

From its first post to a handful of doctors in 1994, ProMED-mail has mushroomed into an e-mail list with more than 30,000 members, who give it eyes and ears around the world. The challenges that confronted the system early on — such as worries about e-mail access in remote locations — have proven less daunting than anticipated. “Now you have Internet cafés in Afghanistan,” says one of ProMED’s three co-founders, Stephen Morse of the Columbia University Mailman School of Public Health in New York. But new challenges, including the threat of bioterrorism, crop up all the time.

ProMED’s grassroots origins and its reliance on volunteers have been vital to its success. Today, it is a worldwide system that has had a huge impact in the halls of power and in the corridors of hospitals. It circulates reports on animal, plant and human diseases and toxins from all corners of the globe. The list posts information from a range of sources: on-the-ground observers, media stories and government reports. And its 26 expert moderators add guidance and context to the reports. As volunteers, they are free to act independently, enabling ProMED to coerce governments and even international bodies into action.

ProMED’s members have helped to diagnose everything from anthrax in Ugandan hippos to severe acute respiratory syndrome (SARS) — which was first publicly reported through a ProMED post last year. Veteran disease fighter Donald Henderson at the University of Pittsburgh confirms ProMED’s influence. When he was advising the US government during the anthrax attacks of 2001,



Henderson says that his team relied on two sources of information: CNN news and ProMED. “We are the CNN of outbreak reporting,” says Jack Woodall, another of the system’s founders.

Spreading the word

Woodall’s own story is closely tied to that of ProMED. Born in China to a family descended from British missionaries, Woodall spent three-and-a-half years of his childhood in a Japanese internment camp during the Second World War. He passed the time there by collecting plants, butterflies and other insects. When Woodall and his parents were released, they moved to England, where Woodall studied entomology. He then got a job in Uganda and retrained as a

virologist. His new work reminded him of his childhood pursuits in the camp. “It was absolutely fascinating to study diseases spread by insects,” Woodall says. “We collected mammals, birds and everything that moved.” After a stint in Puerto Rico, Woodall eventually ended up in Geneva working for the World Health Organization (WHO). That’s where he convened the meeting that laid the foundations for ProMED in 1993.

Woodall’s co-sponsors for the meeting were two leaders from the Federation of American Scientists: Morse and Barbara Hatch Rosenberg, a molecular biologist at the State University of New York at Purchase. Experts at the event were concerned that there was no good way to spread the word about disease outbreaks. E-mail was just gaining a



ProMED-mail's updates on the 1995 outbreak of Ebola in Zaire (left) won it a wider audience and helped alert doctors in the field in Africa (above).



Staying in touch: the architects of ProMED-mail, (from left) Stephen Morse, Jack Woodall and Barbara Hatch Rosenberg, have helped keep the world alert to disease outbreaks.

foothold on the scene, and the group took a gamble that it would eventually become more widespread. A health communications system called SatelLife agreed to host ProMED, and the founders sent out their first e-mail report to 40 people on 19 August 1994.

By that time, Woodall had transferred to New York, where he was heading up the state's arbovirus laboratory. The state was a bit strapped for cash, and it was difficult finding money for all the scientific studies Woodall had planned to do. But rather than being daunted by his low finances, Woodall made good use of his time. His lab had its own computer and an e-mail address, so Woodall became the first director of ProMED-mail.

Morse credits Woodall with guiding ProMED through its early days. Woodall would send media reports out to the list, and he and other volunteers read and posted every report that came into ProMED — a tradition that still holds today. Morse and others filled in for Woodall during vacations. "It was a real mom-and-pop operation," Morse says. Over the first year, the list grew

slowly. "I remember being thrilled when we went from 40 people to 500," Woodall says.

But ProMED didn't truly begin to take off until the following year. In May 1995, an Ebola outbreak erupted in Kikwit in the Democratic Republic of the Congo (then called Zaire). People searching for news of the outbreak on the Internet stumbled across ProMED postings on the disease and signed themselves up to the list. By the end of the year almost 3,000 new members had joined. From there, membership has shot up to today's 30,000 subscribers. "It just grew and began to take on a life of its own," says Larry Madoff, an infectious-disease doctor at Brigham and Women's Hospital in Boston, Massachusetts, who has edited ProMED since 2002.

Early warning

ProMED's mass subscriber base has given it global reach and influence. In May 1996, a ProMED subscriber watching television news in Manila posted a report about a huge cholera outbreak in the Philippines. ProMED disseminated the report more than two weeks before the Philippines allowed the WHO to report the outbreak.

Over the years, ProMED has continued to beat local authorities to the punch in disease reporting. For a case in point, take the SARS outbreak. The first public report of the epidemic appeared on ProMED on 10 February 2003, when a ProMED subscriber passed along news of a mysterious epidemic in Guangzhou, China. "An acquaintance of mine ... reports that the hospitals there have been closed and people are dying," wrote Stephen Cunnion, an epidemiologist retired from the US Navy. The post appeared more than a month before the Chinese government joined the international SARS investigation.

ProMED says that this on-the-ground reporting pushes governments to disclose more information than they would like. At

times, this has caused friction with health authorities and with the WHO. But Woodall thinks that, ultimately, ProMED has helped the WHO more than it has hurt. "The WHO is happy, because it can go to a country and say, look, this has already been on ProMED, you might as well just admit it," says Woodall, who now directs the Nucleus for the Investigation of Emerging Infectious Diseases at the Federal University of Rio de Janeiro in Brazil.

So far, ProMED has managed to survive without paying Woodall or any of its other staff. Since 1999, the service has been officially housed at the International Society for Infectious Diseases in Boston. The software giant Oracle hosts the website, which runs on donations. ProMED also runs an Internet funding drive every year, but there are no subscription fees.

Independent spirit

ProMED's founders don't want to go corporate, or attach the system to a government organization. "We're freer to report what we see than official organizations, which have political constraints on what they can say," says Madoff. But this leaves ProMED in a perpetual state of impending insolvency. "We're always running out of money in 11 months," says Woodall.

Despite these financial difficulties, ProMED hopes to expand its reach during the next decade. To gain more on-the-ground observers, it is planning regional collaborations with organizations in east Africa, the Mekong Basin area of southeast Asia, and with states of the former Soviet Union. ProMED has also set up a project to assign geographical coordinates to each report, enabling editors and users to draw links between scattered reports.

In addition to its expansion, ProMED is navigating a host of new challenges, including difficult issues raised by bioterrorism. A report on an Ebola outbreak in a Manhattan hospital, for instance, could incite public panic — and a false alarm could cause psychological and economic damage. Madoff says that ProMED is aware of these issues. Editors weigh several factors before deciding whether or not to post a report, provide moderators' context for the information, and notify users if the news turns out to be wrong. "We take our responsibilities very seriously," Madoff says.

After all, ProMED's reputation has been a key part of its success. During the next ten years, it hopes to widen its net without losing its carefully cultivated accountability and accessibility. ProMED's backers know they are walking a fine line — but they're not the kind to shy away from a challenge. ■

Erika Check is Nature's Washington biomedical correspondent.

♦ www.promedmail.org

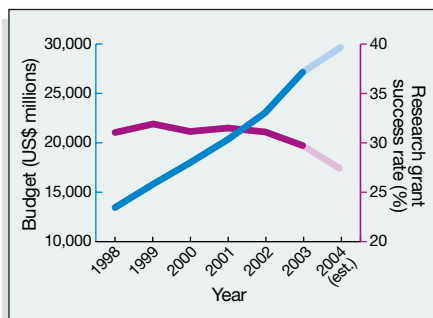
David versus Goliath

Resources are shifting from small labs led by one researcher to large teams with expensive equipment. But has the rise of big biology gone too far? Erika Check investigates.

James Watson and Francis Crick are often cited as the ideal role models for modern team science. Watson was a physicist who crossed over into biology, Crick a zoologist who soon turned to genetics. In the early 1950s, the pair shut themselves in a laboratory in Cambridge, UK, to solve the structure of the basic molecule of life — DNA. When they emerged, they reported a discovery that transformed biology into an information-based science — the precursor of systems-focused biology.

Today, funding agencies around the world are trying to replicate the conditions that allowed Watson and Crick to succeed. They are spending vast resources on multidisciplinary centres aimed at bringing biologists together with chemists, physicists, mathematicians and others, in the hope that new interactions will bring fresh insights to biological problems. Funding agencies are also investing more in technologies and tools, instead of research projects, in a bid to build huge treasure troves of information.

Scientific leaders agree that collaborative projects can produce results that would be impossible for specialized individuals working alone to achieve. But some are worried that the systems-biology revolution, which focuses on the interaction between cogs in the machine, instead of on the cogs themselves, has undermined the foundations of discovery-based science. They wonder if money spent on technology would be better spent on training scientists to think through complex problems. And they are asking whether large projects waste money in bureaucracy, and dilute creativity by forcing a focus on specific diseases. Is there any basis for these anxieties? Or are today's reforms



simply taking biology out of the Stone Age?

The debate is particularly heated in the United States, where a recent doubling of federal funds for biological research was accompanied by a decline in successful grant applications, suggesting that there is a shift towards larger projects (see chart). Concerns about such moves can be heard in Europe as well, but the more fragmented nature of funding sources there means that the trend is less dramatic and may even be reversing. China on the other hand is weathering harsh criticism from some of its leading scientists over its tendency to tackle research in the same way it approaches dam building: on a massive scale.

In the United States, the National Institutes of Health (NIH), the world's largest biomedical-funding agency, is going through a revolution under director Elias Zerhouni. He took the helm in 2002, just as the agency's budget was completing a five-

The multidisciplinary approach of Francis Crick and James Watson (right) helped inspire 'big biology' centres such as the Wellcome Trust Sanger Institute (above). The falling success rate for funding of individual projects at the NIH despite a rising budget (left) emphasizes the trend.

year doubling. Zerhouni declared that the NIH needed to do a better job of translating its research into cures. "Biomedical research is at an important turning point that may require new strategies," he said at his confirmation hearing in April 2002.

Thinking big

Now that the doubling of the NIH's budget is complete, there are some indications that large-scale science has benefited more than traditional, investigator-initiated research. One closely watched barometer is the 'success rate' — the percentage of grant applications that receive funding. In lean times, success rates drop, and individual investigators feel the pinch as they have a harder time getting funding. But success rates for research project grants have dropped overall since the beginning of the budget doubling, from 31% in 1998 to a projected 27% next year, according to NIH data. And success rates for new investigators starting research labs have also dropped slightly, from 24.9% in 1998 to 24.1% in 2003, down from a peak of 25.9% in 2000.

So why are success rates dropping despite



that no single investigator can do everything alone, and that large, team-focused grants are essential tools to solve some problems. But, he adds, new investigator grants, known as R01s, are essential because they allow young scientists to stake a claim for themselves. “R01s enable energetic, young principal investigators to look at things from a fresh perspective,” Grakoui says.

Zerhouni insists that the NIH is not abandoning the individual investigator. The important trend to note, he says, is that success rates per investigator have risen from 30.5% in 1996 to 34% in 2003, even as overall annual rates have fallen. This means that more individuals who apply for funding eventually receive it. “Our intent is to keep the portfolio balanced and I don’t see a significant shift towards agency-initiated or large-scale science,” Zerhouni says.

All mapped out

But some biologists are also worried about a more fundamental shift. As part of his vision to move more basic science into the clinic, Zerhouni implemented a plan a year ago called the ‘Roadmap’ to fund more projects that focus on technologies, team science and clinical research. Although it will eventually account for only 1% of the NIH budget, it seems to have propelled other changes in funding priorities.

For instance, in August the agency updated its peer-review criteria to accommodate interdisciplinary, translational and clinical projects better. The ‘significance’ category used to omit any mention of clinical research, but it now asks reviewers to consider whether “scientific knowledge or clinical practice” will be advanced by the funding application. And in October, the National Institute of Mental Health completed a plan to shift its focus from basic research to projects that target specific diseases.

Zerhouni agrees that the Roadmap is causing fundamental changes at the NIH — but he argues that such changes are long overdue. “We need to tackle complex biology in ways that favour our ability to solve these problems, and we need to tackle the issue of bench to bedside,” he says.

Zerhouni is not alone. In Europe, research leaders such as geneticist Rudi Balling, scientific director of the German Research Centre for Biotechnology in Braunschweig, are lobbying for more ‘big science’ projects. “There is no way around interdisciplinary work,” Balling says. “We need to build networks so that large investments can be shared.”

But this is more difficult to do in Europe than in the United States, where one agency controls the whole research budget. Jacques Remacle, a scientific officer in the European Commission’s research directorate, says that,

in Europe, research funding for biology comes from many different sources, requiring agencies to team up to compete with the United States on large projects. “Only a few countries, such as Britain and Germany, can afford large initiatives,” he says.

Some even sense a retreat from big biology in Europe. For instance, German federal funding for the National Genome Research Network, set up in 2001, will decrease from the €180 million (US\$237 million) that covered its first three years to a projected €135 million for the next three. Hans Lehrach of the Max Plank Institute for Molecular Genetics in Berlin laments the decline. “Investments for large-scale projects are moving backwards and now most of the funding is going back to classical research,” he says.

China, on the other hand, has the opposite problem. Last year, Premier Wen Jiabao initiated a strategy for the next 15–20 years called the National Mid-to-Long Term Plan for Science and Technology. This advocates funding for large ‘megaprojects’ costing anywhere from hundreds of millions to billions of dollars.

But Chinese scientists around the world are warning that this may not be the best way to spend research money. They allege that science is still too intermingled with politics in China, and that money may be wasted on bad projects. They want the government to build up the country’s scientific talent and resources by investing in more small-scale research first, before committing to huge, top-down projects.

“Organizing big projects without a sufficient number of high-quality researchers ... will undoubtedly lead to the waste of resources,” writes Mu-ming Poo, director of the Institute of Neuroscience at the Shanghai Institutes of Biological Sciences, in a recent supplement to *Nature* in Asia (see *Nature* 432 (Suppl.), A18–A23; 2004).

Central perks

The worldwide commitment to big biology stems partly from the success of the Human Genome Project, which spurred the growth of large sequencing centres everywhere from the Wellcome Trust Sanger Institute near Cambridge, UK, to the RIKEN Genomics Sciences Center in Yokohama, Japan. At the same time, biology is now asking questions about complex networks of cellular signals that require groups of specialists taking a team approach. A number of large research centres have been built for this purpose, such as the US\$150-million James H. Clark Center at Stanford University in California, which was funded by a private donation.

But in the United States at least, the shift has had as much to do with politics as the needs of research. The idea of doubling the

the rise in funds? Part of the reason is that more of the cash is going to research centres, which support groups of investigators who agree to work on a designated problem. By the end of 2005, the NIH’s spending on research centres will have grown by 131% since 1998 compared with only 97% for research project grants.

These indicators are concerning some scientists, especially those just starting out, who rely on individual project grants to set up new labs. “It is definitely worrisome,” says Arash Grakoui, a virologist and immunologist who in July began building his first lab at Emory University in Atlanta, Georgia. “As a new investigator, it is even a little discouraging.” Grakoui says that he is increasingly realizing

“There is no way around interdisciplinary work. We need to build networks so that large investments can be shared.” — Rudi Balling



STANFORD UNIVERSITY

Group therapy: the James H. Clark Center at Stanford is a new multidisciplinary institute that was built to tackle some of biology's toughest problems.

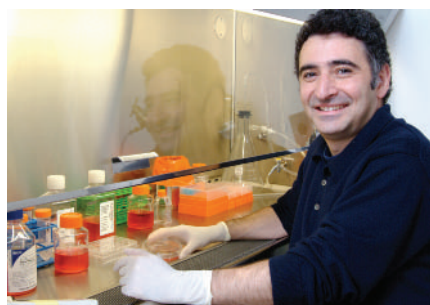
NIH's budget was supported by a broad coalition of universities, scientists, research institutes and patient advocates. These groups lobbied tirelessly during the early and mid 1990s for the rise. The genome project helped them to convince lawmakers that science was proceeding in new ways, and that the NIH needed to grow so that US science could keep pace.

"If we had wanted to stay with R01 science, we probably wouldn't have gotten past a \$13-billion or \$14-billion agency," says April Burke, a lobbyist for biomedical research in Washington DC, who was heavily involved in the doubling. "It was the fact that we were changing that allowed policy-makers to say, 'Yes, I will invest in what you're doing.'"

Team spirit

Many basic biologists are supportive of the shift towards team science, and agree that researchers should think more about medicine. Nobel laureate Paul Berg, a biochemist at Stanford University, is among them. He says that 20 years ago, graduate students weren't interested in medicine. "But today," Berg says, "you see graduate students in basic science coming to clinical seminars. The expectation is that somewhere, somebody's brain is going to recognize that what they're doing applies to some disease." He agrees that a shift too far towards clinical research would be bad for the NIH, but he doesn't think that this has happened yet.

Signs abound that the team mentality is taking hold. While young scientists all hope to earn individual support to start their careers, they are acutely aware of the need to



Left out: Arash Grakoui worries projects centred on individuals are being sidelined by big biology.

form teams, to gain access to expensive equipment, and to take advantage of where the funds are flowing.

"You have to think in terms of the best investment strategy, where you try to diversify a little and apply not only for R01s, but also for other opportunities," says John Wherry, an immunologist who in January will start his first lab at the Wistar Institute in Philadelphia, Pennsylvania. Wherry has watched as higher and higher merit scores — the numbers assigned by review boards to grant applications — have become necessary to win an R01 grant, while the requirement for centre grants has remained stable. "If it's possible to get involved with some larger submissions that's going to provide a better opportunity for junior people right now," he says.

Although many see the shift as a positive thing, the real problems may still lie ahead. At the end of its budget doubling, the NIH has enough money to spread around many different kinds of projects. And a \$1.7-billion cash infusion for biodefence research has also

boosted the agency's bottom line. But the years ahead don't look so flush. The biodefence bonanza is over, and after years of double-digit percentage increases in its budgets, the NIH received just a 2.8% increase for this year, and will get a 2% increase for 2005.

A massive budget deficit and a clamp-down on all spending outside of homeland security and defence mean this situation will persist for the foreseeable future. This is what keeps some science advocates up at night — they realize that it is easier to trim new investigator grants than to cancel funding already promised to large research centres. "We certainly are concerned," says Paul Kincade, an immunologist at the Oklahoma Medical Research Foundation and current president of FASEB, a coalition of American biomedical research societies based in Bethesda, Maryland.

It's difficult to know what all these changes mean for scientists who want to follow in Watson and Crick's footsteps. The team approach promises to advance the work of basic scientists, but researchers need the freedom to pursue new ideas about biology — even if it's not clear how they will have an immediate impact on patients' lives. With its coffers relatively full, the NIH is in a position envied around the world: it can fund both large-scale and small biology. But if US funding for science drops, the agency may be forced to make some tough choices. "As things tighten up, as they seem to be doing, we need to protect individual investigator-initiated science," Kincade says. ■

Erika Check is Nature's Washington biomedical correspondent. Additional reporting by Federica Castellani.

Tackling grade inflation in US universities

Solutions could include reporting the class average and ranking departments by results.

Sir—Your Editorial “Against grade inflation” (*Nature* 431, 723; 2004) raises an important question in US higher education that affects both the efficacy of our assessments of student performance and the credibility of those assessments.

Back when I was on the other side of the lectern, my impression was that a grade was a statement of relative academic performance. A “C” was actually defined in the academic catalogue as “average”, along with “A = excellent”, “B = above average”, “D = below average” and “F = failure”. These days I see that the academic catalogue defines a “C” as “satisfactory” and a “D” as a “minimum passing” grade. This change reflects the lamentable fact that grades no longer hold any real contextual meaning.

I believe that change is long overdue. If professors are too nervous about appeasing students and parents to stick to the definition of “C” as average, then student grades should be reported, as they were at my undergraduate institution, along with the average for the class. So instead of receiving a naked “B”, a student might receive a “B – , 3.4/3.62” — indicating that this student was actually 0.22 grade units below the class average of 3.62, despite receiving a grade higher than a “C”.

This approach would work well for universities’ internal use, but what about external evaluation? Perhaps US colleges and universities could be ranked using some system analogous to the impact factor of journals, a ranking that might be derived from the performance of its

students on standardized national exams.

To avoid potential difficulties, such as ranking a department with a superb record within a mediocre university, it would seem sensible to perform this ranking on a departmental as opposed to a university-wide basis.

In this way, a student could be more or less objectively evaluated by a graduate school or prospective employer as having a “3.44/3.12 grade-point average from a 1.76-ranked US university chemistry department”. Wouldn’t that be a more scientific approach to measuring student performance in the academic crucible?

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Grade inflation: students seek it, funders reward it

Sir—Your Editorial (*Nature* 431, 723; 2004) identifies a serious problem, but modifying the use of student evaluations is only a partial solution. Students actively ‘shop’ for courses with grade inflation.

If money is allocated to departments on the basis of the number of students enrolling on a course, as it is at my university, then grade inflation is rewarded with additional funding.

We need to make courses compete on content rather than grading. One way to level the playing field would be to include students’ percentile ranks in each class (for instance, 90% if only 10% of students in the class had higher scores) in their transcripts, along with traditional grades. How this might affect competition among universities is an interesting question.

R. Ford Denison

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Grade inflation keeps the customers happy

Sir—As your Editorial notes (*Nature* 431, 723; 2004), grade inflation is indeed real in the United States, and not only at private institutions. The evidence isn’t anecdotal: the data (see www.gradeinflation.com) show that grade inflation is omnipresent at community colleges and at both public and private four-year schools.

Your solutions to this problem could be easily implemented. However, getting things turned around would require university leaders willing to buck the ‘keep the customer happy’ ethos on US campuses. Unfortunately, such leaders are few and far between. Grade inflation is a symptom of an overarching problem in higher education: a failure of university leadership to have the courage to preserve the integrity of US higher education.

Stuart Rojstaczer

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Journals must cooperate to defend biosecurity

Sir—In publishing the Letter to *Nature* by D. Kobasa and colleagues, “Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus” (*Nature* 431, 703–707; 2004), *Nature* has endorsed a publication that reports the creation of an influenza strain with increased virulence (at least in mice) based on the molecular structures of one of the deadliest diseases of the twentieth century. This will surely bring both benefits and risks to biosecurity.

Following a discussion of the problems arising from the publication of biosecurity-sensitive data, scientific journal editors came together and agreed that this issue deserves attention and that some general measures should be in place (see *Nature* 421, 771; 2003). Since then, most leading journals, including *Nature*, have introduced procedures to deal with this issue.

However, there are good reasons to believe that these individual journal-specific procedures are inadequate, in that they give the least restrictive journal the ultimate control over sensitive biosecurity information. Looking at the tremendous impact the outcome of the editor’s decision may have on the public, informed democratic participation in the decision-making process must be a requirement. At present, none of these journals release information on the risk–benefit analysis undertaken in specific cases to allow independent assessments.

As long as clear guidance from legislators is missing, the policies followed by these scientific journals will remain non-transparent, possibly inconsistent and subject to political bias. Let’s hope that the initiatives being pursued by several countries, including the United States (www.aaas.org/spp/post911/agents), to negotiate biosecurity guidelines for scientists, will lead to workable and publicly accepted principles regarding all aspects of research, including the publication of research results.

A necessary first step towards transparency could be the publication of the local biosafety committee’s reasons for giving approval, the biosafety measures taken and the editor’s risk–benefit assessments.

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The blame game

Who will pay for the damaging consequences of climate change?

Myles R. Allen and Richard Lord

Last year one of us (M.R.A.) wrote a Commentary in *Nature*, asking if it would ever be possible to sue anyone for damaging the climate¹. At the time, this was largely a thought experiment, as the scientific and legal arguments were too immature to provide a satisfactory answer. But in this week's issue, Peter Stott, Daithi Stone and M.R.A. provide some scientific justification for revisiting that question². Their paper considers how much human influence on climate could be 'to blame' for the southern European summer heatwave of 2003.

After the cold and wet summer of 2004, this might seem a bit of a mug's game. But for scientists, the contrast between these two summers graphically illustrates the role of chaos and chance in climate. Stott and colleagues show that using risk and probability analysis makes it possible to quantify a link between external inputs (such as greenhouse gases) and specific weather events.

Linking responsibility for damage directly to greenhouse-gas emissions has always been a taboo subject in the climate-change debate. But some of us may, unwittingly, already be paying the costs of adapting to climate change. As emission targets for greenhouse gases will take decades to have any discernible effect, the critical issue for most people today is not climate treaties, but working out who will pay the costs of adaptation³, and compensation for those who cannot adapt.

Playing the odds

Stott and colleagues do not suggest that 'but for' past greenhouse-gas emissions the 2003 heatwave could not have occurred, nor that such heatwaves will now happen every year. The immediate cause of the heatwave was a persistent anticyclone over northwest Europe⁴ — no one could sensibly claim that greenhouse gases caused that particular anticyclone. Instead, Stott *et al.* argue that we have loaded the weather dice — that human influence on climate has increased the risk of an anticyclone causing a heatwave like that of 2003 by around a factor of four. They also estimate with higher confidence (a 10% chance of being wrong) that more than half the risk of such a heatwave was due to human influence, primarily greenhouse gases. That may sound frustratingly convoluted, but uncertain estimates of relative risk are all that will ever be scientifically defensible.

If a dice is loaded to come up six, and it comes up six, there is a clear sense in which the loading 'helped cause' the result. If the loading doubles the chance of a six, it follows that half the sixes you get are caused by the loading. The question of 'which sixes?' is meaningless. If you throw a one, it does not prove the dice was not loaded, so the 2004 washout has almost no effect on conclusions about 2003. We say 'almost', because if we now get 20 years of summers like that of 2004, the scientific community might have to revise its



The 2004 lawsuit by eight US states and New York City aims to force five power companies to reduce carbon dioxide emissions.

conclusions about the loading in 2003, and a number of people would have egg on their face. Like any scientific statement, Stott and colleagues' claims are falsifiable. But they represent a conservative assessment of what can be said in the light of current knowledge.

Let us assume subsequent studies focusing on smaller scales, such as the Paris region, also find a substantial role for human influence in loading the dice (what studies of smoking and cancer call the 'fraction attributable risk') for the weather that summer. This is by no means guaranteed. On the one hand, the smaller the scale considered, the less predictable will be the relationship between global greenhouse-gas concentrations and local temperatures, and therefore the values of attributable risk might fall¹. On the other hand, local temperature anomalies in 2003 (see map, overleaf) were more extreme than changes to the regional-scale seasonal average, and correspondingly less likely to have occurred by chance in an unmodified climate⁵, which might push up values of attributable risk. So it could go either way. This matters, because the area-averaged temperature for southern Europe studied by Stott and colleagues didn't kill anyone, unlike the temperature in Paris.

The French authorities estimate that the 2003 heatwave caused more than 14,000 'excess deaths' nationwide⁶. The number for which the temperatures were the principal cause of death would be lower, but could still run into thousands. Suppose it is confirmed, at a reasonable level of confidence, that past greenhouse-gas emissions doubled the risk of these local temperature anomalies. This would surely meet or exceed the threshold at which a court might conclude those emissions were, in a loaded-dice sense, likely to have been a 'legally effective' cause of death and hence that some victims might have grounds to claim compensation against those responsible for the emissions⁷. There are complications: mortality rates normally go up in the winter, so it might well be argued that some of the elderly victims would have died in the next few months anyway, although the spike in August 2003 exceeded even the annual cycle⁸.

Dangerous games

The 1992 United Nations Framework Convention on Climate Change commits its signatories to stabilize greenhouse-gas concentrations at levels that would "prevent dangerous anthropogenic interference with the climate system". Evidence that past greenhouse gas emissions are already, in a statistical sense, killing people makes the convention's goals seem more urgent than ever. But it also makes it clear that they are unachievable, at any conceivable stabilization level, if we apply the word 'dangerous' to vulnerable groups such as poor elderly Parisians. The convention makes greenhouse gases sound like any other regulated pollutant — provided we keep levels below some internationally specified limit, all will be well. They aren't, and it won't. Emitting greenhouse gases is dangerous. So are many other productive activities, such as building hydroelectric dams. But those killed or rendered homeless when a dam bursts generally have some legal redress, ideally from those who benefit from the energy produced by the dam. What will happen in the case of those killed or rendered homeless by climate change?

It is important to keep the risks in perspective — Stott *et al.* conclude that 2003 was still an improbable event, even allowing for human influence on climate, although the risks are changing rapidly (their model suggests that by mid-century, 2003 would be an average summer). So you have to be particularly

AP PHOTO/K.WILLENS

unlucky and vulnerable to be personally at risk from climate change today. Risks to property may turn out to be much more significant in the short term. The reason is that something has to happen (such as a heatwave) to kill people — the dice has to roll. It could happen next year, or it could not happen for a decade. But in any well-developed financial system, it only takes the discovery that the dice is loaded to reduce the value of property immediately. If you are buying property in the Maldives, it will be hard to argue you are an innocent victim of climate change when the sea level rises, and you would be well advised to take this into account when agreeing a price. Consequently, it is the current owners who may bear the cost of future climate change — even though they won't be around when the islands finally submerge.

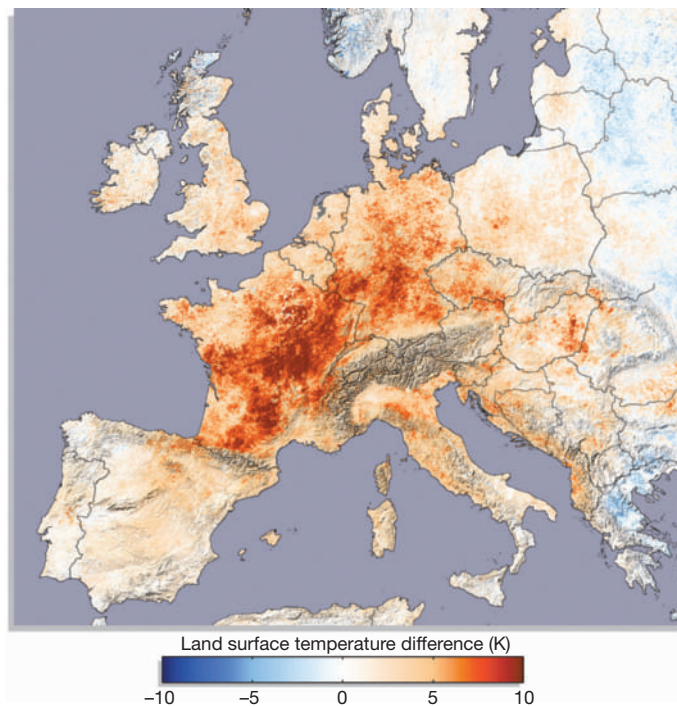
Talk of stabilization targets and 'dangerous climate change' focuses attention on the relatively distant future, but in fact, much of the financial adjustment to climate change may be happening right now, under our noses. As with the deaths from the heatwave, individual cases will be hard to pin down. Is it hard to sell the house because of rumours of increasing flood risk, or because the neighbours built that hideous conservatory? Is it hard to sell the farm because of concerns over water supplies for the next 20 years, or because cotton prices happen to be low? In the world of markets and perceptions, things can happen much faster than the pace of climate change itself. As markets race to adjust residential, commercial and agricultural property values to reflect new risks due to climate change, there will be winners as well as losers, but it seems unlikely that the winners will rush to give away their windfall, whereas we can be sure the losers will be looking for someone to blame.

Cause, chaos and the courts

They may not have to look very far. Preliminary studies suggest that a substantial fraction of our current elevated level of carbon dioxide might be traced to products produced, sold or used by only a few dozen major companies⁹. Until now, most of the legal focus has been on regulations, but litigation in relation to greenhouse-gas emissions is increasingly likely, and has already started. Over this summer, eight US states and New York City filed a lawsuit against five US power companies for their contribution to climate change.

A key objection raised by defendants in

such cases¹⁰ is that the plaintiffs cannot demonstrate actual harm arising from greenhouse-gas emissions. This situation may be changing as the science of attribution matures, although the study by Stott and colleagues shows that — even for highly unusual events — it will almost always be impossible to say that 'but for' greenhouse-gas emissions this event would never have occurred. Notably, English law takes a more flexible approach to questions of causation, with a 2002 case in the House of Lords (*Fairchild v. Glenhaven*) suggesting that "material increase in risk" may sometimes be an appropriate test.



Land surface temperatures for summer 2003, relative to the summers of 2000–04. From NASA's Moderate Resolution Imaging Spectrometer, courtesy of R. Stockli.

Other legal questions about whether emitters should have foreseen damage, and their fault or negligence, will present formidable hurdles to claimants. If European summer temperatures continue to evolve as Figure 1 in Stott and colleagues' paper suggests, it will become increasingly hard to argue that any resulting damage was unforeseeable. But fundamental issues will remain. As there are no direct observations of what the European climate of 2003 would have been like if greenhouse-gas emissions had not occurred, quantifying human contributions to risks will always depend on computer simulations. How would a court view this kind of evidence? In principle it should be admissible: computer simulations are not unknown in the courtroom, however unnerving climate modellers may find it to have the tools of their trade being picked over by skilled defence lawyers.

Litigation and regulation on climate change will become increasingly intertwined,

as court decisions will be influenced by regulatory policy and regulators will increasingly have to consider how their decisions affect liability. In the United States, the power generators sued have argued that government policies are responsible for controls on climate change, whether pursued domestically or internationally, and so this precludes private lawsuits¹⁰ (what one might call the 'relax, the federal government is in control' defence). In Britain, a private law of right to compensation can only be excluded by a regulation or statute that clearly has this effect. Current UK policy is to persuade emitters to do something rather than nothing, even

if that something does little to avoid damage or maximize its mitigation. So, it seems unlikely that implementing and complying with current and proposed emission regulations will take away anyone's right to sue.

How can current emitters best respond? To begin with, they could seek clarification of what participation in a carbon emissions trading scheme actually means. As the law stands at present, such participation should probably not be seen as an insurance policy against future liability, although many may like to think of it that way. In responding, governments will have to consider the implications carefully. If they grant indemnity from liability to encourage participation in emission trading, are they prepared to take on that liability themselves (apart from the obvious 'moral hazard' issue of transferring the cost from today's emitters to tomorrow's

taxpayers)?

Although these ideas may seem far-fetched now, we could one day see Californian farmers suing member states of the European Union for authorizing emissions that threatened the security of their water supplies. Whatever the weather in Europe next summer, we can be sure that the argument over who pays for the cost of climate change is here to stay. ■

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In from the cold

Mary Somerville's contribution to science has been undervalued.

Mary Somerville and the World of Science

by Allan Chapman

Canopus: 2004. 176 pp. £12.95

Collected Works of Mary Somerville

edited by James A Secord

Thoemmes Continuum: 2004. 9 vols, £750

Patricia Fara

The educational reformer Maria Edgeworth urged women to study chemistry, although she made it sound like a glorified form of cookery by saying it “demands no bodily strength” and “applies immediately to useful and domestic purposes”. But as Allan Chapman reveals in his lucid biography of Edgeworth's close friend Mary Somerville (1780–1872), it was the male astronomer John Herschel who ventured into the kitchen. Herschel told Somerville that he had purloined a dead lobster to examine the spectral pattern of its phosphorescent glow. Somerville herself was a talented mathematical physicist endowed with organizational abilities that enabled her to run a substantial household while carving out her reputation as one of Victorian England's most famous scientists. As Edgeworth quipped: “Her head is among the stars, her feet are firm upon the Earth.”

Then, as now, Somerville intrigued commentators because she was an unusual woman, but Chapman — an eminent historian of astronomy — quite rightly stresses her scientific significance. As evidence of her research capabilities, she published original articles in several leading journals, including the *Philosophical Transactions* of the Royal Society. But her achievements were not an indication of equality. Excluded from the male enclave of the Royal Society, she delegated to her husband the privilege of reading out her paper (although the society's fellows did pay Somerville the dubious tribute of installing a marble bust of her in the hallway).

Although Somerville was barred from universities and official societies, she did more than most men to promote scientific awareness. Her books interpreting the latest scientific ideas were far from being simplistic popularizations, and tackled complex contemporary problems in mathematical physics. Pierre Laplace, the self-styled ‘French Newton’, respected Somerville so highly that he chose her to explain his mathematical astronomy to the British scientific community, and her book *Mechanism of the Heavens* converted Laplace's five dense volumes into a scholarly yet accessible vision of



Star turn: Mary Somerville did more than most men to promote astronomy and physics.

the universe. Fifteen years later, she sold more than 15,000 copies of her book *On the Connexion of the Physical Sciences*, an eloquent review of cutting-edge science that consolidated as well as disseminated the latest discoveries.

Chapman has produced a new pocket-size biography of Somerville, and its timing reflects a growing academic interest in her work. Unknown to Chapman, James Secord, a science historian at the University of Cambridge, was simultaneously preparing a splendid edition of Somerville's collected works. Benefiting from illustrations and independent introductions, the nine volumes present facsimile versions of all her publications, including five previously unknown mathematical solutions found through some nifty sleuthing work in the archives. The range of topics displays the breadth of Somerville's accomplishments: some volumes bristle with formulae, whereas others discuss, for example, tiny marine organisms, Antarctic birds, comets, volcanoes and education. The final volume is reserved for her “personal recollections” and provides a fascinating insight into Victorian networks as well as her own experiences.

Despite her prominence during her long life, Somerville has been doubly marginalized. As a woman, she has been set apart from mainstream scientists and studied as an oddity; and as an interpreter, rather than an originator, of scientific knowledge, she has been relegated to the sidelines. Both Chapman and Secord insist that she be brought centre-stage and considered within the full context of nineteenth-century science and its impact on society. Secord's efforts will make that process of integration much easier, and the size and scope of his collection testify to Somerville's importance.

His publisher's welcome initiative to make her original texts accessible to scholars signals a shift in the type of question that historians ask about science's past. Instead of concentrating on great discoveries, theories and inventions, they are now analysing how science has come to be so dominant. Science is successful, they argue, not simply because it is right, but because people say it is right. This reassessment is vital not only for Somerville herself, but also for the larger project of demonstrating that science's history is about far more than equations, instruments and great men. To understand how science came to form the backbone of our modern world, we need to describe not only what happened inside laboratories and libraries, but also what happened in the outside world.

Shifting the focus has demonstrated that, although in the past women made different contributions from men, their work was far from insignificant. Chapman argues that Somerville could only have flourished in what he repeatedly refers to as Britain's unique “grand amateur scientific environment”. One striking counter-example is Emilie du Châtelet, Somerville's French Enlightenment equivalent who had translated and commented on Isaac Newton's work much as Somerville did for Laplace.

Before the twentieth century, there were so few outstanding scientific women that it is tempting to make each one a special case, although many of them shared three characteristics: brains, wealth and an accommodating partner. Nevertheless, from a modern perspective, it is dispiriting to recognize that women believed in their own inferiority. “I have perseverance and intelligence but no genius,” wrote Somerville, “that spark from heaven is not granted to the sex.” ■

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A family affair

Consanguinity, Inbreeding and Genetic Drift in Italy

by Luigi Luca Cavalli-Sforza,
Antonio Moroni & Gianna Zei
Princeton University Press: 2004. 320 pp.
\$79.50, £51.95 (hbk); \$39.50, £26.95 (pbk)

Walter Bodmer

More is known about natural populations of humans than of any other species. There are records, usually publicly available, about the geographic distribution of populations, their demography and patterns of migration and mating, and often about how these vary with time. These are the factors that affect the population distribution of genetic variability, generally subsumed under the definition of population structure.

When the distribution of genetic variation can be explained by the population structure, it can be assumed that natural selection has not played a role in determining the distribution, at least within the limits of detectability. This effect should be the same for all 'neutral' genetic variation, so when variation is significantly heterogeneous, as pointed out nearly 40 years ago by Luca Cavalli-Sforza, this is evidence for the existence of natural selection. The determination of population structure is therefore an important goal for human population genetics. This has been emphasized recently by the need for carefully chosen control populations for disease association studies involving large numbers of DNA polymorphic markers. Small relative risks may easily be masked, or apparently created, by hidden population structure, leading to numerous false positive or negative associations.

Cavalli-Sforza and his colleagues in Italy have spent more than 50 years studying human population structure and its consequences. This extraordinary book, packed with detailed information, is their account of this work.

The initial stimulus was the opportunity to access the extensive records of the Catholic church on dispensation for marriages between close relatives (consanguineous marriages). These data allowed estimates of the frequency of marriages between cousins, for example, or between an uncle and his niece, and through that the extent of population inbreeding and its effects. The first studies made use of parish records in the Parma valley, but later ones used the Vatican's archives covering the whole of Italy.

The world-wide variation in consanguineous marriages is surprising: there is a high frequency in many Arab Muslim populations, even though the Koran condemns marriages between close relatives, as does the Judæo-Christian tradition. The dispensation data do not always record marriages



Kissing cousins? Italian marriage registers have revealed the frequency of human inbreeding.

between more distant relatives, so they may underestimate the level of inbreeding by as much as a factor of five.

However, a great deal of information can be obtained from the relative frequencies of the different types of matings, and the way they change over time. These frequencies are affected by the difference in age between husband and wife, by the tendency in a more or less stable population for a woman to move to her husband's locality, and by whether women rather than men try and arrange marriages. Marriages between an aunt and her nephew are much less common than those between an uncle and his niece.

For marriages of equal lineage, for example between first or second cousins, it is the number of males and females in the lineage that affects their relative frequency: the more males there are, the less the effect of migration and the commoner the type of marriage. Perhaps counterintuitively, increasing the population size may raise the proportion of consanguineous marriages, as long as migration does not increase. In the first quarter of the twentieth century there was a slow increase in the rate of consanguineous marriages, but this decreased as industrialization and improved transport and communication increased the rate of migration.

Detailed theoretical modelling, especially computer simulation, shows that the frequencies of the different types of mating between relatives are essentially as would be expected if they occurred by chance. Inbreeding does not seem to be specifically avoided, as is often presumed, because of its negatively perceived effects in uncovering deleterious recessives. However, marriages between brother and sister are prohibited in most cultures, and there is a similar avoidance of matings between members of the same kibbutz in Israel.

Surnames behave as surrogate genetic markers on the Y (male-determining) chromosome. Cavalli-Sforza and colleagues use extensive data from telephone directories to obtain a distribution of surnames throughout Italy. They use this to estimate the key parameters of population structure and show that these, on the whole, match those obtained using genetic markers. These and other studies suggest that most of the common blood-group-determined polymorphisms can be explained by genetic drift in the absence of natural selection, at least in the populations studied. The authors conclude that, even with the extensive DNA data now available, there is no conclusive evidence that natural selection affects common polymorphisms.

This is surely not the case. The variation observed in the HLA alleles, for example, is so unevenly distributed along the gene, matching regions of functional significance and mostly due to non-synonymous substitutions, that this alone, following Cavalli-Sforza's own suggestions, must be due to selection. The fact that most polymorphisms detected by random screening of DNA sequences of genes are synonymous, or occur in introns, points in the same direction.

A careful reading of this remarkable book will yield much more information. More diagrams and histograms would have helped readers to digest the mass of data, and better copy-editing would also have been useful.

In commenting on the value of parish registers in England for a more satisfactory study of surnames, the authors say: "Here is a task for an ambitious and dedicated organiser." It is hard to imagine that anyone could be more dedicated than Cavalli-Sforza, Moroni and Zei.

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Science in culture

A hobbit-forming image

Peter Schouten's painting of *Homo floresiensis*.**Martin Kemp**

The 'wild man of the woods' (*Homo silvestris*) regularly featured in Renaissance and later illustrations of 'primitive men'. He tended to be rather hairy, but less so than a typical ape. Before Darwin's time, these primitive men did not routinely exhibit ape-like features; they were basically like us, only ruder in aspect.

By contrast, post-darwinian 'missing links' have been portrayed as just that, exhibiting bodily and facial features stranded somewhere between contemporary apes and modern humans. Darwinian 'ape-men' are almost invariably portrayed as miserable and destitute, living in grinding discomfort, clearly waiting desperately for evolution to happen — even if not in their lifetimes.

These portrayals of prehistoric humans are now joined by the image of *Homo floresiensis* painted by the Australian artist Peter Schouten, which achieved front-page coverage in international newspapers towards the end of October. 'Lifelike' reconstructions meet a basic human instinct to see what someone looked like, whether it be William Shakespeare or a Stone Age labourer. Such images flourish in the popular domain but tend to be denigrated within science, although few palaeontologists can ultimately resist the temptation to visualize extinct creatures in living flesh and blood.

Scientists will readily recognize that Schouten, like any artist relying largely on bones, had to make some key assumptions, not least with respect to fleshy and surface features, including secondary sexual characteristics. For a historian of images, a series of questions arise about the 'character' with which the envisaged figure is endowed. We cannot portray any figure without giving it some kind of definite persona, however subjectively its characteristics may be read by different spectators. The features that speak most powerfully to us — the



eyes, nose and mouth — are among the most speculative.

Schouten's diminutive man is hairier than a modern human. He is male — early women rarely appear outside a family or tribal context. He returns from the hunt, with tethered trophy and multi-purpose digging stick; wooden weapons have long since been standard equipment for hairy men. We almost never show primitive men carrying bunches of fruit or doing anything less than

macho. He does not quite share the standard look of down-and-out melancholy, but seems to manifest a certain stoic acceptance of his condition. In any event, he does not look like a bundle of fun.

For the historian, the circumstances behind the production and use of such an image are integral to understanding both why it looks the way it does and how it is viewed. Schouten has specialized in such reconstructions, having illustrated *A Gap in Nature* by Tim Flannery, director of the South Australian Museum. Flannery suggested to Richard 'Bert' Roberts of the University of Wollongong that Schouten be asked to produce a picture of Flores Man. The resulting painting was purchased jointly by the university and the National Geographic Society, and the society then acquired the image rights (their television channel will air a programme on the discovery early next year). The image was released to the public as soon as the original scientific papers appeared in *Nature* (431, 1043–1046, 1055–1061, 1087–1091; 2004) on 28 October.

The illustrations published in *Nature* remained impeccably within what would be widely accepted as 'scientific': an evolutionary tree in two formats; graphs of relative brain and body sizes and of relative skeletal dimensions; sets of osteological photographs (one on the cover); and a computer-generated section. The main article by Peter Brown and co-workers is rigorously sober in its cataloguing of the skeletal remains, with judicious extrapolations about the hominin's diminutive stature.

But the battered skull and bony fragments do not stick in our memory in the way that Schouten's skilful painting does. The process of discovery and publication has thrown up an instant icon that will be very hard to dislodge. We can change our mind about recorded facts, but a potent image, for good or for ill, tends to become indelible.

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Burning questions

Tending Fire: Coping with America's Wildland Fires

by Stephen J. Pyne
Shearwater: 2004. 226 pp. \$25

Ross Bradstock

Major wildland fires are spectacular to look at, and the drama continues into their political and social fallout. It is a truism that fires shape the nature of ecosystems and biodiversity, but they can also shape human institutions and values. This is the central thesis of Stephen Pyne's latest book, *Tending Fire*, in which he attempts to get to grips with fire not only as a biophysical problem, but also as a socio-political one.

Pyne argues that the United States' problem with fires is symptomatic of a deeper clash over the values that society places on its wildlands. The problem is exacerbated because the public institutions traditionally involved in managing fires are not capable of dealing with this debate. Part of Pyne's solution is a call for a greater input from the humanities into the study of wildland fires. An understanding of the history of fire policy and management, and of the psychology and art of fire, among other things, could help those responsible for fire management to function with more self-awareness, he argues.

The public ownership of wildlands will endure, Pyne surmises, because there is an implicit consensus about their value as ecosystems that have been spared the worst

ravages of exploitation. But the debate will continue about the appropriate level of intervention and institutional arrangements needed to manage fire. The monolithic control of fire policy and management on public lands by "imperial" institutions (such as the US Department of Agriculture's Forest Service) is starting to unravel. Pyne foresees an era of cooperative devolution, in which localized decision-making and hands-on action is shared among public and private players. He highlights as a model the role of the Nature Conservancy in the United States, which is both an active manager and a community-level broker of fire management ideas and solutions.

Tending Fire is a condensation of general themes and arguments — a summation of Pyne's larger works. It focuses on the great



California burning: US wildfire policy is caught up in a greater clash over the value of wildlands.

public wildlands of the western United States, which are the scene of catastrophic wildfires wrought in equal part by nature and by the putative failings of the people charged with their management. The "pyric transition" — the switch from 'natural' biomass fire to the industrial use of fossil fuels — is briefly recapitulated. Pyne recounts its progression from "free-running" fire, experienced by indigenous peoples, to European colonial exploitation (including overgrazing, clearing, logging and mining), the creation of reserves, and the advent of bureaucratic command and control.

The core of the book is an account of the four fundamental pillars of fire management: suppression, 'let burn', prescribed fire, and fuel treatment. Pyne counsels that relying on any one alone is doomed to failure, as history has shown. They all have their place in solving the fire problem, but in what particular mix? Beyond noting that different mixes are likely to be required in different ecosystems at different times and places, Pyne offers no comprehensive solution.

His vision, focused on ponderosa pine forests, is heavily qualified. Forceful arguments, such as the need for mechanical thinning and the re-introduction of surface fires, are tempered by caveats. For example, wildfires are inevitable and serve useful ecological purposes, and anyway, the best solution depends on the locality, as crown fires may be required in chaparral and high-altitude conifer forests. At times the juxtaposition of solutions is breathtaking: devolution of planning responsibility to the community on one hand, with increased government regulation of urban design on the other. Pyne does, however, paint a slick picture of climate change and the consequences of burning fossil fuels, and of the international pressures that may be brought to bear on US

fire management to reduce emissions.

Ultimately, *Tending Fire* succeeds as a visceral and widely accessible account of the problem of wildfires. Pyne does not solve it but lays it out in all its maddening, self-contradictory splendour. His attempts to sketch a way forward, although useful, amplify the paradoxes and the choices available. Wisely, he counsels that, at best, both art and science can illuminate the consequences of differing choices but are not surrogates for decision-making.

The book concludes with a call for a biological theory of fire. This is a noble effort but the sketch offered is disappointing. The nostrum that fire is a by-product of life (biomass) is useful, but falls short. Fire is frustrating because we do not properly understand how it works at the spatial and temporal scales at which we confront it. Physical and ecological knowledge is shackled within micro-scale, reductionist paradigms that are inadequate for understanding fire and its consequences on a larger scale. Coping with fire is about understanding and manipulating forms of heterogeneity and biophysical feedbacks that we have barely grasped and that are not amenable to 'bottom-up' scientific enquiry. It is about recognizing that fire poses both risks and benefits at several levels. Compromises and trade-offs must be engineered accordingly, but the functional knowledge required for effective management is lacking. Fire is a transcendent phenomenon in both biophysical and socio-political senses. *Tending Fire* contributes to our awareness of this, but there is a long road ahead.

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"More inclusive and better reading than similar attempts by science journalists." Maxine Singer, *Nature* **422**, 809 (2003).

Correction

In his review of Graeme K. Hunter's book *Light is a Messenger* (*Nature* **431**, 1037–1038; 2004), Kenneth C. Holmes stated that there was an error in Figure 0.2 in the book. In fact, this figure is intended to show a polychromatic, rather than a monochromatic, diffraction experiment, in which case the Bragg reflections are correctly displayed.

Wee beasties

How a tiny observation turned out to have a controlling influence.

Paul Nurse

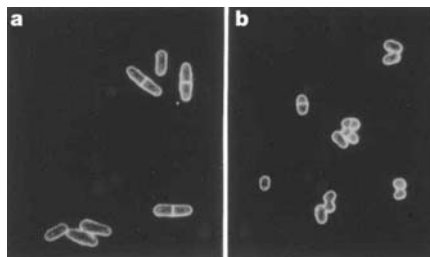
A major turning point in my scientific life involved not only a group of individuals (important as they were for what happened), but also a microorganism — or to be more precise, a mutant microorganism. The time was 1974 and the place was Murdoch Mitchison's laboratory in the zoology department at the University of Edinburgh. Mitchison had been interested in the cell-division cycle since the 1950s, working mainly with the fission yeast *Schizosaccharomyces pombe*. He recognized that the length of this rod-shaped, single-celled organism was a good marker for how far the cell cycle had progressed, which made fission yeast a convenient model for cell cycle studies. It was this microorganism which transformed my scientific life.

The previous year, I had been a graduate student in Norwich studying amino-acid pools in fungi. This project required me to nurture a rather temperamental Beckman amino-acid analyser, and I often found myself keeping this machine company throughout the night — just to make sure it kept running! During these long night vigils, I read numerous research papers to keep me awake and two from Lee Hartwell attracted my attention. These described the isolation of temperature-sensitive cell-division-cycle (*cdc*) mutants in budding yeast (*Saccharomyces cerevisiae*), which failed to progress through the cell cycle when grown at a high temperature. Immediately, I decided that I wanted to do something similar and, being familiar with Mitchison's work, thought fission yeast would be a good organism for this project. I contacted Mitchison, who was enthusiastic about me coming to Edinburgh, but wisely suggested that I should first learn and become familiar with genetic techniques. So I went to Urs Leupold in Bern, where he and his colleague, Peter Munz, taught me fission-yeast genetics. I finally arrived in Edinburgh at the start of 1974, with my newly acquired (but rather limited) genetic knowledge, and started isolating *cdc* mutants in fission yeast.

However, the isolation procedure was very laborious and so progress was very slow. First, temperature-sensitive mutants (those unable to form colonies at a high temperature) had to be identified by replica plating yeast colonies from a low to a high temperature.



The author (left) and Pierre Thuriaux in more relaxed settings, 1974. Below, the 'wee' mutant (panel b) in comparison to other yeast cells.



Next, these mutants were screened visually using a microscope to find colonies that contained elongated cells, which indicated that they could not complete their cell cycle and divide, although they continued to grow. By the end of the year I had only found approximately 30 *cdc* mutants.

I devised an enrichment procedure to try and speed things up. I centrifuged cell populations through a density gradient to separate large, elongated cells from normal-sized cells. The larger cells, some of which would be *cdc* mutants, should make it to the bottom of the gradient, and these fractions were plated out. After isolating a few mutants this way, something strange happened. There was a clump of cells in the middle of the microscope field that seemed to be smaller than normal. My first response was disbelief — how could such small cells have got to the bottom of the gradient? This thought was slowly replaced by another one — what was the significance of these tiny cells? Fission yeast cells usually divide at a constant cell length, so if these cells were shorter than normal, this must mean that they had divided at a small size. This was only possible if cells had progressed more rapidly through their cycle, dividing prematurely

before they had grown to their normal size.

I called over my friend and colleague, Pierre Thuriaux, who was visiting from Bern. He was aware of my limited genetic skills and gently suggested that my plates were contaminated with another microorganism. It was another day before we and Mitchison were convinced that it was really fission yeast, albeit a small one. During our conversations we recalled a seminar from a colleague in the department of genetics, Henry Kacser, on the control of metabolic flux. Kacser had argued that although control was distributed through all steps of a metabolic pathway, some steps could be more rate-

limiting than others. Was it possible that this small-sized mutant (soon to be called 'wee' to reflect its Scottish origin) had a defective gene for a major rate-limiting step in the pathways controlling cell-cycle progression? This simple interpretation turned out to be correct when it was eventually demonstrated that *Wee1* regulates the cyclin-dependent kinase that acts as the major engine of the fission-yeast cell cycle.

What lessons did I learn from this turning point in my career? The first was the importance in biology of letting the organism you are studying guide you in the right direction to solve your problem. By using microscopic visual inspection, I was not being too restricted in my search for mutants. If something unexpected turned up, I could see it. This allowed mutants that no one had imagined existing before to be observed and thought about, opening up new avenues of enquiry. Serendipity was given a chance to play a role, allowing nature to constrain and guide the infinite possibilities that occur to the human mind.

A second lesson was the importance of spending time trying to understand the real biological significance of observations made on cells and organisms before undertaking detailed investigations of the molecular mechanisms involved. Understanding the biology properly is as important as the subsequent mechanistic insight.

One final thought — why were the wee mutant cells at the bottom of the gradient? I don't know, perhaps it was because they clump easily or maybe I was just very lucky. ■

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Hot news from summer 2003

Christoph Schär and Gerd Jendritzky

The European heatwave of 2003: was it merely a rare meteorological event or a first glimpse of climate change to come? Probably both, is the answer, and the anthropogenic contribution can be quantified.

The European summer of 2003 was characterized by highly anomalous meteorological conditions¹, and was extremely hot and dry^{2,3}. In the northern parts of the continent, the summer was perceived as beautiful and warm. But in central and southwestern Europe, the heat was prolonged and intense, and the economic and societal consequences were disastrous (as described in Box 1).

Given the heatwave's severe repercussions, the question has arisen whether the summer of 2003 is evidence of man-made climate change. On page 610 of this issue, Stott, Stone and Allen⁴ take a major step towards answering this difficult question. Previous studies had found that recent changes in the European summer climate were consistent with climate-change scenarios^{5,6}, but there had been no attempts at a rigorous attribution of cause and effect. Indeed, because the atmosphere is a chaotic dynamical system, it is impossible to attribute — in a causal sense — an individual episode of extreme weather to changes in atmospheric composition. Nevertheless, it is feasible to estimate the probability or risk of occurrence of a certain weather event under natural and modified climatic conditions. This is the avenue taken by Stott and colleagues.

Using one of the leading global climate

models available, the authors derive the probability distributions of European summer temperatures for two sets of climate simulations, each covering the period since 1900. The first set accounts for the past effects on climate that were due to variations in solar and volcanic activity, as well as to man-made influences (including increases in greenhouse-gas concentrations). The second set mimics a natural climate by prescribing natural factors alone. Stott and colleagues then calculate the changed risk of extremely hot summers that is attributable to past anthropogenic emissions of greenhouse gases, using a comparison of observed and simulated summer temperatures to account for uncertainties in man-made warming and natural variability. They find, at a confidence level of greater than 90%, that more than half of the risk of 2003-like extreme European summers is attributable to human influences on the climate system.

Methodologically, Stott and colleagues⁴ use an approach developed for detecting global climate change and attributing causes to the changes identified. There is long experience with such studies, all of which find that a significant anthropogenic contribution is required to explain the observed global climate records of the past 30–50 years^{7,8}. The new study fits into these results, as the

probability of extreme heatwaves must change as mean temperatures increase. The details of the analysis are rather complex. But the basic interpretation of the main result is comparatively straightforward: anthropogenic warming shifts the statistical distribution of summer temperatures towards warmer conditions, and this has a dramatic impact on the chance of temperatures exceeding some threshold out in the upper tail of the temperature distribution.

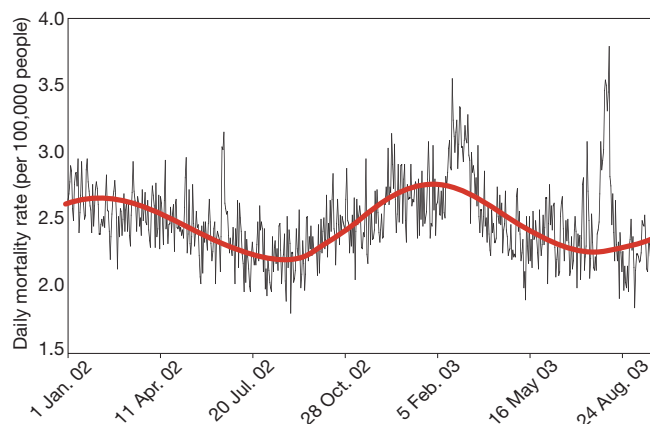
What about the limitations of the new work? We will mention two. First, Stott *et al.* address the whole summer of 2003 (and not the extreme heatwave in early August), and all of continental and southern Europe (not the much smaller central European region where the heatwave was most intense). Consideration of shorter-term and smaller-scale heatwaves will require higher computational resolution⁹, and will need to take the complexities of land-surface processes into consideration^{1,3}. Accounting for these factors is a challenge. Second, representing natural climate variability is a general difficulty in studies attempting to attribute causes to particular effects. Stott *et al.* show that their model appropriately represents the spectrum of continental-scale European climate variability on interannual to inter-decadal timescales. But more detailed

Box 1 Impacts of the heatwave

According to reinsurance estimates, the drought conditions during the summer of 2003 caused (uninsured) crop losses of around US\$12.3 billion, while forest fires in Portugal were responsible for an additional US\$1.6 billion in damage. The European electricity markets reacted erratically to increases in demands, as power plants had to curtail production owing to the lack of cooling water, and electricity spot prices soared beyond €100 (\$130) per MW h. In the Alps, many glaciers underwent unprecedented melting, and the thawing of permafrost led to a series of severe rock falls.

But it was the unusual number of deaths during 1–15 August that caught the headlines. Estimates based on the statistical excess over mean mortality rates amount to between 22,000 and 35,000 heat-related deaths across Europe as a whole¹¹. In France the mortality rate increased by 54% during those two weeks, and the increase was statistically significant in all 22 French regions and for all age groups above 45 years¹².

The figure, reproduced from ref. 13, shows the daily mortality rate in Baden-Württemberg, Germany, over a period of 20 months, and puts the August



2003 heatwave in context. Total daily mortality data are in black, with the mean seasonal evolution in red. Notable features are the seasonal cycle, with higher mortality in winter; a heat-related mortality peak in June 2002; the

effects of an influenza outbreak in February–March 2003; and the striking peak in August 2003, due to the heatwave, which caused 900–1,300 extra deaths in a population of 10.7 million people.

C.S. & G.J.

studies will be needed to corroborate this conclusion, as there are large uncertainties in the estimates of natural climate variability—derived from both models and observations⁸.

Nonetheless, Stott and colleagues' work constitutes a breakthrough: it is the first successful attempt to detect man-made influence on a specific extreme climatic event. Such events are among the most notable features of a changing climate, not least given their impact on human affairs. Another article in this issue, by Allen and Lord (page 551)¹⁰, discusses how refined analyses might lead to liability claims for costs incurred by climatic shifts. The advent of such 'attribution studies' might profoundly affect the course of international negotiations on ways to mitigate, adapt to and ultimately pay for the consequences of climate change. ■

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Information science

Quantum errors corrected

Andrew Steane

The phrase 'quantum error correction' might sound like a technical fix to a device that ought to be working better. But it is in fact a fascinating piece of fundamental physics with powerful implications.

Quantum error correction is a central concept of quantum information science and is almost the only thing a quantum computer would need to do if it is to work properly. It gives me great pleasure to say that it has now been implemented, in its most simple form, in a laboratory experiment reported by Chiaverini *et al.*¹ on page 602 of this issue.

It is surprising indeed that irreversible changes in quantum systems can be corrected. A correcting machine should first gather information from the faulty system, but for a quantum system this would cause the unavoidable disturbance associated with observation. We need to engineer an observation in such a way as to disturb the error, not the stored information, and to learn what the error is after the influence of our observation. Chiaverini and colleagues¹ have done exactly that.

Traditionally, 'Alice' is the protagonist in any quantum information story. Imagine that Alice wishes to preserve an atom's quantum state in the presence of noise. The state can be thought of as a spin, or rotation, about an axis oriented in three dimensions. (This is a short-hand for a pair of hyperfine levels in the electronic ground state of a ⁹Be⁺ ion.) It is described by the notation $a\uparrow + b\downarrow$, where a and b are complex coefficients, and $\uparrow$ and $\downarrow$ are two spin directions. We assume that Alice does not know what state her atom is in,

because if she did she could circumvent the whole problem by writing on a Post-it note — "Don't forget: a is 0.8, b is 0.6". Such cases are of no use for quantum computing.

Alice cannot examine the atom, because this would disturb its state. She cannot generate copies of it (that is, prepare further atoms in the same state) because no method to do that is physically possible (the 'no-cloning' theorem, which if broken would lead to various contradictions involving non-local correlations). However, Alice can cause her atom to interact with two others so that the group of three is now in a state described by $a\uparrow\uparrow + b\downarrow\downarrow$ (I have simplified things a little here, because Chiaverini *et al.*¹ in fact used an elegant, closely related, encoding, but this one is easier to describe). This is akin to radio operators' use of 'alpha' and 'bravo' for 'A' and 'B' to reduce errors: a longer symbol, here the three-atom state $\uparrow\uparrow\uparrow$ in which all atoms have spin-up, is used to encode a shorter one, the state $\uparrow$ (and similarly $\downarrow\downarrow\downarrow$ encodes $\downarrow$).

The atoms are now left alone for a while. Suppose the error processes mostly reverse the orientation of individual atoms. Then the state is likely to be corrupted into $(a\uparrow\uparrow + b\downarrow\downarrow)$ or $(a\uparrow\downarrow + b\downarrow\uparrow)$ or $(a\downarrow\uparrow + b\uparrow\downarrow)$, or any combination of these possibilities, through quantum superposition. In the experiment¹, to enable them to make a quantitative study, the team

introduced an artificial error of known size, such that the probability of a spin-flip was p per atom. Alice's task is to manipulate the group so as to bring a given atom, say the first one, to the state originally stored ($a\uparrow + b\downarrow$) — but she must not learn what that state was, or she will have disturbed it. Her operations must somehow reveal or react to the error, without learning the original message.

There is a way to do this: measure pairs of atoms, to determine whether their spins are aligned, without allowing information about any individual atom to be revealed. A general method for this, using quantum logic gates, was discovered in 1995. Such measurements 'project' the state, so that instead of a superposition of the possibilities listed above, the atoms must adopt just one of those possibilities. This is the unavoidable disturbance associated with the act of observation, but in this case it is engineered to make the state better defined and thus easier to correct. Furthermore, the correction can now be completed, because Alice can deduce from her measurements which, if any, atom has an inverted spin.

In Chiaverini and colleagues' experiment¹, these operations were performed by moving atoms in ultra-high vacuum along a segmented array of ion traps, each 100 micrometres in dimension. To control the rotations of the atoms, the team extended a technique of their own invention: a pair of laser beams with a precise frequency difference between them to drive the oscillations of the atoms. In this new work, the laser pulse was made to act on three atoms simultaneously, such that the force cancelled when all spins were aligned, and had the same magnitude for all states in which the spins were not all aligned. This performed most of the encoding or decoding in one step, greatly simplifying the (nevertheless still very demanding) experiment.

Although other experiments in liquid-state nuclear magnetic resonance^{2,3} have demonstrated encoding and correcting operations for this and larger codes (more atoms), in these the signal was halved for each further spin introduced, and the fundamental ingredient of either individually measuring, or else reinitializing, the state of the extra spins was not available. Chiaverini *et al.*, however, have demonstrated all of the ingredients of quantum error correction in a single experiment. Their results are summarized by the formula $P \approx q + 2.6p^2|ab|^2$, where P is the probability that the final state was wrong, and $q \approx 0.22$ is the contribution from imperfection of the apparatus. For technical reasons, only a small range of initial spin states could be prepared, but this does not diminish the main achievement. It is also notable that for $p > 0.25$ a net suppression of noise (that is, $P < p$) was attained.

There are several natural steps to take next. One is to replace the rotation by a

fundamentally irreversible decoherence process, induced for example by scattering a photon from the atoms. As long as this projects the spin state without relaxing it, it could be corrected by the same code. Another is to achieve repetitive correction, something not available in the experiments so far. It would also be very valuable to see a five-atom or a similar code in action, correcting general

errors, including relaxation, rather than only rotation about a known axis.

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Archaeology

Greater expectations

Peter W. Stahl

Evidence of unexpected complexity in an ancient community in Uruguay is a further blow to the conventional view of prehistoric development in marginal areas of lowland South America.

Archaeological research often reveals unexpected results. This is common in South America, especially when archaeologists venture off the beaten track to explore unfamiliar areas. However, our surprise is also a product of our preconceptions. Recent work in the lowlands of tropical South America clearly bears this out, with discoveries of prehistoric complexity in unforeseen places and/or times^{1–6}. On page 614 of this issue, Iriarte *et al.*⁷ present another example of precocious development in a hitherto little-explored and under-appreciated area. The authors refer humbly to their results as unexpected; but given the profusion of surprises elsewhere, why would they be unexpected in the first place?

The conventional view suggests that little of archaeological importance can be expected of 'marginal' areas — those areas geographically distant from a great Andean "center of inventiveness and social development"⁸. Although the origin of this idea can be traced

directly to the *Handbook of South American Indians* (HSAI), conceived in 1939, and published between 1946 and 1950, its roots are certainly deeper. With state-of-the-art knowledge at his disposal, HSAI editor Julian Steward^{8,9} cobbled together a summary of cultural history in South America that used a now-outmoded belief in cultural evolution, culture areas and trait diffusion; environmental determinism; a sketchy archaeological record; and an underestimation of the effects of European conquest on native populations.

How does one understand the bewildering complexity of the humans of pre-Columbian South America? In his tentative historical summary, Steward subsumed indigenous South America under a ranked scheme based on sociopolitical and religious patterns, and shared or missing cultural elements. He sketched out the historical-developmental implications of his classification, putting Central Andean civilizations at the top, and descending through circum-

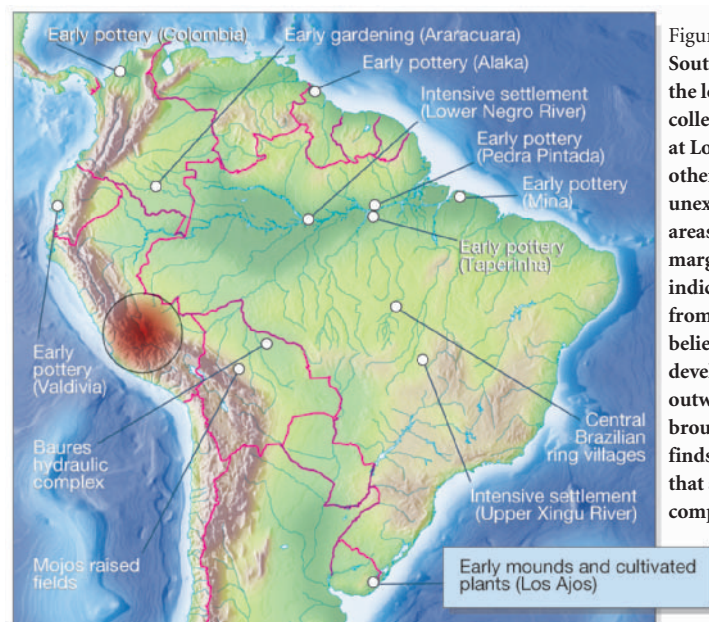


Figure 1 Marginal or not? South America showing the location of Iriarte and colleagues' investigations at Los Ajos⁷ and some other examples^{1–6} of unexpected discoveries in areas once considered marginal. The red circle indicates the general area from which Steward^{8,9} believed cultural developments spread outwards — a view brought into question by finds in 'marginal' areas that are earlier or more complex than expected.



50 YEARS AGO

One hundred, or even fifty, years ago there was far less to understand... but certainly it becomes every day more difficult to find scientists whose interests are wide enough to assist the advance by helping the specialists to understand one another. There is in the Library of Trinity College, Cambridge, a collection of letters illustrating the value of such help. They were written between the years 1830 and 1860 by... Michael Faraday and William Whewell, the great discoverer seeking advice from the most learned scholar of his day. Faraday was in difficulties with his experiments on electrolysis. He needed new terms to describe what he was doing... Whewell..., in a letter dated May 5, 1834, strongly advises the terms 'Anode' and 'Cathode'. The letter continues: "If you take *Anode* and *Cathode* I would prefer for the two elements resulting from electrolysis the terms *Anion* and *Cation*, which are neuter participles signifying *that which goes up* and *that which goes down*; and for the two together you might use the term *ions* instead of *Zetodes* or *Stechions*." And Faraday replies ten days later to say that he has taken Whewell's advice and ends his letter: "I am quite delighted with the facility of expression which the new terms give me and I shall ever be your debtor for the kind assistance you have given me." From *Nature* 4 December 1954.

Caribbean/sub-Andean, to tropical forest peoples, and with 'marginal' tribes at the bottom. Steward proposed an Andean centre of development with unlimited agricultural potential (a factor considered essential for establishing large, sedentary populations), from which cultural traits had diffused into other portions of the continent. According to the theory, in recipient areas, cultural elements were varyingly adopted or lost for historical or ecological reasons. In particular, marginal areas fared poorly — some areas were so far removed from the Andean centre that little was passed on. Besides, local environmental conditions were supposed not to be conducive to prehistoric agriculture in these marginal areas, thus necessitating a constant nomadic quest for subsistence.

Although few would buy into these ideas today, Steward's culture history has had an enormous impact on archaeological interpretation, both academic and popular. Using this perspective, 'traditional Indians' are conceptualized as having made ancient ecological adaptations that allowed them to survive relatively unchanged since deep time. In areas or periods where archaeological facts

exceeded predisposed expectations, external migrations or influences were sought in the form of lost Japanese fishermen, brief intrusions from centres of 'high culture', or the late result of European manipulation. In a curious twist, those who argue that the seeming marginality of some tribes is more probably the recent product of global events are chastised for ignoring the ecological success of indigenous populations or for acting as accomplices to environmental degradation^{10,11}.

We can surely do better than this today. Iriarte *et al.*⁷ demonstrate that we actually do (Fig. 1). Their investigations at Los Ajos in the La Plata Basin of southeastern Uruguay reveal a large formal village plan, consisting of mound and plaza features, at a time (more than 4,000 years ago) and in a place where conventional wisdom would not have expected them to exist. Moreover, subsequent occupation, intentional remodelling, settlement planning and village size indicate both a permanence and a density of population previously unthought of for this area. Innovative analyses of plant microfossils and starch grains extracted from stone tools yield evidence for the early exploitation of maize, squash, beans and root crops in an area that was long considered non-agricultural, at least for prehistoric populations. The findings add another example to a long and growing list of pre-ceramic agricultural sites throughout the world, which reminds us that farmers do not necessarily need ceramic pots. Pottery shards have traditionally been the interpretive touchstone among archaeologists working in South America, and conventional practitioners have slavishly devoted technical and methodological energy to their retrieval and study.

The study by Iriarte *et al.*⁷ not only rejects much of the interpretational baggage carried by generations of archaeologists, but also exposes the potential for prehistoric culture in grasslands and wetlands, which were historically viewed as marginal areas^{1,12}. Marginality and atrophied development are part of a flawed historic perspective. Our expectations for indigenous achievements should be greater.

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Cytoskeleton

Spindle saga

Eric Karsenti

It's generally been thought that, during cell division, only proteins are necessary to assemble the machine that segregates chromosomes. But a new molecular requirement has been discovered.

Arguably the most striking structure a cell can produce is the spindle — a fine meshwork of filaments that carries out a fundamental task in cell division. Before one cell can make two, it must duplicate its DNA; the replicated chromosomes must then be separated and pulled to opposite poles of the cell, which splits into two. The mitotic spindle is the machine that segregates the duplicated chromosomes. It is built from an array of protein tubes — the microtubules — that assume a precise size and bipolar organization.

Several proteins, notably molecular motors and various signalling proteins^{1,2}, are essential for spindle organization and function, but no other types of molecule were thought to be involved. That view will now change, however, with the paper by Chang, Jacobson and Mitchison on page 645 of this issue³. These authors have found that a large, branched, polymeric molecule, known previously for its effects on chromosome structure, is also essential for microtubules to be organized into a functional spindle.

Work over the past 15 years has shown that spindle assembly results from the self-organization of chromosomes, microtubules and molecular motors⁴. While this is happening, some microtubules attach to specialized structures on chromosomes, the kinetochores; both kinetochores and microtubules are required to move chromosomes to the spindle poles⁵ (Fig. 1a). This self-organization process involves the collective action of microtubule proteins, motor proteins and signalling proteins^{1,2}; Chang *et al.*³ now find that another type of chemical — poly(ADP-ribose), PAR — also has a key role. ADP (adenosine diphosphate) is a nucleotide, akin to the basic building blocks of DNA; ribose is a sugar molecule.

It is known that ADP-ribose is added in the form of a long branched polymer to some proteins, and in particular to histones — nuclear proteins that help to package chromosomes into a compact state called chromatin. This addition of PAR to histones is done by a family of enzymes called poly(ADP-ribose) polymerases (PARPs); the

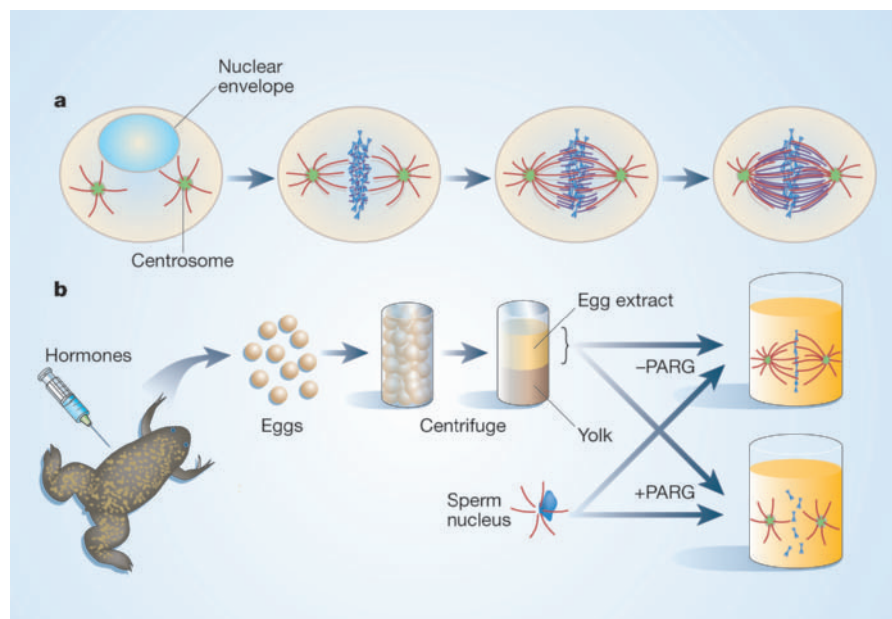


Figure 1 Spindle assembly — proteins and poly(ADP-ribose). a, As cells enter mitosis, the nuclear envelope disappears and microtubules (red), nucleated by centrosomes, become very short. Chromosomes (light blue) condense; new microtubules (purple) start to grow and become stabilized around chromosomes. Molecular motors (not shown) help to organize the microtubules into two antiparallel arrays — the spindle. b, This process can be recapitulated with frog egg extracts into which sperm nuclei containing centrosomes are added. Now Chang *et al.*³ have found a crucial role for poly(ADP-ribose) (PAR). They show that if poly(ADP-ribose) glycohydrolase (PARG) is added to the extract to prevent the addition of PAR to proteins, only asters form around chromosomes, and a bipolar spindle cannot assemble.

polymer can be removed by a poly(ADP-ribose) glycohydrolase (PARG). So, the level of PAR on histones at steady state depends on the relative activity of these enzymes⁶. During the period between mitoses (nuclear divisions), the addition of PAR to histones results in a change in chromatin structure.

At first sight, all of this has no obvious connection with the mitotic spindle. What led Chang *et al.*³ to suspect that PAR might also be involved in spindle assembly was that all PARPs were known to be present in one way or another in mitotic spindles, and that the cellular concentration of PAR seems to increase significantly during mitosis. But that did not necessarily imply that this protein modification is involved in spindle assembly.

So Chang *et al.* turned to the well-characterized *in vitro* system of frog egg extracts to address this question. These extracts are obtained by crushing frog eggs in a 10,000g gravitational field for 10 minutes. After sperm nuclei are added, functional spindles form. To test the role of PAR in spindle assembly, Chang *et al.* added an excess of the protein that antagonizes PAR addition (PARG) to such extracts, and examined the spindles formed. The effect was dramatic: instead of nice bipolar structures, they saw microtubules organized as two asters (star-shaped structures) on each side of the chromosomes (Fig. 1b). The microtubules remained dynamic, but could not interact properly at the equator. The paper does not say whether such spindles could segregate chromosomes, but the effect on their structure is so marked that this is unlikely.

Chang *et al.* have thus unveiled a new molecular system that is involved in spindle assembly. However, at the moment it is utterly unclear what PAR does. One should keep in mind that the addition of PAR is, like phosphorylation, a post-translational modification — one that occurs on proteins after they have been synthesized. So to understand how it participates in spindle assembly, it will be essential to identify the spindle proteins that are modified by PAR, and to discover which of these are relevant to PAR's involvement in bipolar spindle formation.

One possibility raised by the authors is that, during cell division, PARPs are activated by phosphorylation, as suggested previously⁷, and that this might lead to the increased addition of PAR to spindle proteins. The polymeric and charged nature of PAR might then help to keep microtubules organized around chromosomes, by providing a large charged matrix. PAR might also act as a signalling device (just as phosphorylation does) to regulate the activity of enzymes or the molecular interactions required for spindle assembly.

An alternative suggestion is that the addition of PAR to chromatin could itself be required for spindle assembly. Indeed, it

seems to me that, in at least some of Chang and colleagues' figures, PAR localizes throughout the chromosomes. This would be in keeping with the known addition of PAR to histones and to kinetochores⁸. A further possibility is that PAR is added both to spindle proteins and to chromatin.

Another question concerns the fact that although the levels of PAR are generally increased during mitosis, they are ten times greater on spindle proteins than on cytoplasmic proteins. That suggests that the relevant enzymes, PARP and PARG, are spatially regulated. This could perhaps lead to a gradient of PAR around chromosomes, analogous to the gradients of phosphorylation and of the Ran protein (a signalling molecule)^{2,9}. Alternatively, PARG might not be active on microtubule or chromatin proteins.

Assembly of the mitotic spindle is a complex process of self-organization involving several enzymatic systems, each of which defines a regulatory module that is often involved in other functions between mitoses. The Ran module, for example, is usually involved in the selective distribution of proteins between the nucleus and cytoplasm, but also coordinates spindle

assembly during mitosis. Now we know that the PAR module, which regulates chromatin structure and function between mitoses, is likewise involved in spindle assembly. It seems that cells are indeed¹⁰ systems in which a given regulatory module can function at different times or under different conditions, to coordinate different aspects of cell organization. ■

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Cell division

The heart of the cycle

Jiri Lukas and Jiri Bartek

To reproduce, cells must copy their genetic material and distribute the replicas into two daughter cells. A self-perpetuating molecular oscillator regulates periodic transitions between these two processes.

Good things come to those who wait — a maxim that is as true for cells as it is elsewhere in life. Dividing cells must wait until their DNA is completely duplicated before they can segregate the replicas. They must also delay the next round of DNA replication until the copies are safely deposited in two newly born daughter cells. The order and completion of these events are crucial; otherwise, chromosomes become unstable, and cancer or premature ageing may result.

On page 588 of this issue, Rape and Kirschner¹ provide a glimpse into how cells ensure the proper timing of such mission-critical events. The result is stunningly simple. The cell-division machinery contains a self-perpetuating engine whose mechanism is based on alternating two intertwined enzymatic activities, namely enzymes that add phosphate groups to other proteins (cyclin-dependent kinases, CDKs)², and enzymes that guide periodic protein destruction³. Although the basic components of this engine were already known (indeed, their respective discoverers were honoured by Nobel prizes in 2001 and 2004),

the new results help us to understand how their periodicity is orchestrated.

Rape and Kirschner's work exploited the known regulation of cyclin A (one of the few cyclin proteins that is truly essential for the vertebrate cell-division cycle) and its roles in initiating cell division (mitosis) and DNA replication (S phase)⁴. In the gap (G1) period between these two phases, cells reduce their concentrations of cyclin A to allow the completion of chromosomal and cell division, and to prepare the ground for S phase (Fig. 1). This reduction is achieved through the destruction of cyclin A, catalysed by the anaphase-promoting complex (APC, also known as the cyclosome)⁵. APC belongs to the family of ubiquitin ligases — enzymes that mark other proteins for destruction by labelling them with chains of a small polypeptide called ubiquitin³. To become active, APC must interact with one of two adaptor proteins, Cdc20 or Cdh1, which physically couple APC with its substrates. APC must also interact with one of the family of ubiquitin-carrier (Ubc) proteins, which bring ubiquitin into the mix.

So far this is reasonably straightforward,

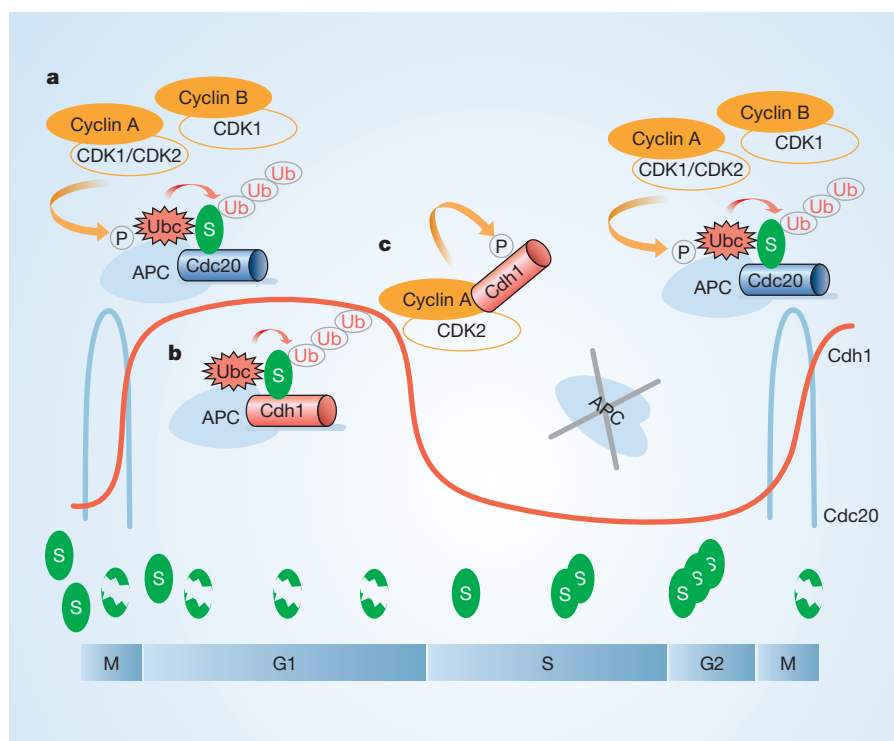


Figure 1 The oscillations of the cell cycle. **a**, Just before a cell's duplicated DNA is separated during mitosis (M), the APC ubiquitin ligase (coupled to the Cdc20 adaptor protein) becomes phosphorylated (P), and thereby activated, by cyclin–CDK enzymes. Together with ubiquitin-carrier (Ub) proteins, the activated APC transfers ubiquitin (Ub) to several substrates (S) — including cyclins A and B — whose destruction (bottom) is necessary to complete DNA segregation. Cyclins A and B thus trigger a chain of events that lead to their own destruction, which is necessary to initiate the new cell cycle. **b**, In the ensuing gap (G1) phase, APC swaps its substrate-binding adaptor from Cdc20 (which is also degraded) to Cdh1. This keeps APC 'on' even without CDKs, allowing complete destruction of mitotic regulators from the previous cycle and guarding against their premature accumulation. **c**, Before S phase begins, Cdh1 is phosphorylated and dissociates from APC. This inactivates APC and allows the re-accumulation of APC substrates required for DNA synthesis and mitotic entry, closing the cycle.

but there's something paradoxical about the behaviour of cyclin A and APC. The problem is this. APC–Cdh1 complexes target cyclin A for destruction, and remain active throughout G1 phase⁶. But cyclin-A-dependent CDK activity is needed to inactivate APC–Cdh1 as G1 phase progresses into S phase^{7,8}. How can cyclin A accumulate to levels high enough to inactivate the enzyme that is triggering cyclin A's own destruction? In broader terms, how can the cell cycle ever proceed beyond the G1–S-phase boundary?

By analysing extracts from human cells that were progressing through the cell cycle, Rape and Kirschner¹ noticed that although many APC substrates were efficiently degraded during the entire G1 phase, the degradation of cyclin A gradually tailed off as cells proceeded through G1. This was unexpected, and suggested that the interplay between APC and cyclin A had a unique component, not shared by other APC substrates. Indeed, through a systematic depletion and re-addition of APC's known co-workers to the cell extracts, the authors found that the degradation of cyclin A was highly sensitive to the concentration

of UbcH10, one of the ubiquitin-carrier proteins.

Rewardingly, further experiments substantiated the special relationship between cyclin A and UbcH10. First, the levels of the UbcH10 protein oscillated during the cell cycle: it was highly abundant in mitosis and early G1 phase, when cyclin A was degraded, but less abundant in late G1 phase, when cyclin A started to accumulate. Second, UbcH10 was itself regulated by the G1 form of APC (APC–Cdh1), and in a different way from other APC substrates. Most strikingly, ubiquitination of UbcH10 required this protein's own enzymatic activity. So, UbcH10 can trigger its own destruction as G1 phase progresses.

These findings naturally raised questions about the purpose and regulation of such self-destruction. Rape and Kirschner observed that although the presence of other APC targets did not interfere with UbcH10 activity as such, it strongly inhibited self-ubiquitination of this protein. So, as long as G1 cells contain some non-degraded APC substrates, UbcH10 (together with APC) prefers to add ubiquitin to them. Only after

these mitotic substrates have been degraded does UbcH10 turn its deadly weapon on itself. The answer to the puzzle, then, is that the carefully timed self-destruction of UbcH10 creates conditions that are permissive for cyclin A accumulation, even when APC remains fully competent to target other substrates (Fig. 2, overleaf).

The discovery of the self-ubiquitination of UbcH10, its regulation by the presence of other APC substrates and its specificity for cyclin A are Rape and Kirschner's key findings¹, providing us with a glimpse into the very heart of the machinery that governs the oscillation between DNA duplication and its partitioning. These results uncover an intrinsic oscillator that controls the periodic alternation of CDK activities and APC activities, the two major driving forces behind the cell cycle, even without any external inputs. The timer is the UbcH10 protein and its amazing ability to sacrifice itself — but only after the daughter cells have received complete copies of the parental genome.

Future studies should establish how universal, and how truly autonomous, this oscillating timer is. It does, for instance, seem to be absent from simple organisms such as yeast¹. It also remains to be seen what, if any, role it has in the rapid cell cycles that occur during embryonic development — or whether it functions when non-proliferating (quiescent) cells re-enter the cell cycle, when mitotic APC substrates will be long gone and therefore unlikely to affect the timing of cyclin A accumulation in the initial G1 phase.

And even this self-perpetuating oscillator can hardly be entirely independent of external signals, from which cells in a multicellular organism cannot be extricated. Indeed, Rape and Kirschner point to one interface with signals that adjust the pace of cell-cycle progression. This provides a back-up mechanism and involves the Emi1 protein, whose accumulation in late G1 phase depends on stimulation by growth factors⁹. Emi1 can inhibit the APC–Cdh1 complex, and Rape and Kirschner show that it couples the extracellular signalling with APC inactivation even if, for some reason, the internal oscillator fails.

One further question relates to the fact that the cell-cycle machinery — including the protein-degradation mechanism — is commonly deregulated in cancer¹⁰. It would be interesting to see whether there are any malfunctions in the UbcH10 timer that might contribute to human disease.

Our knowledge of the oscillation between DNA duplication and cell division has largely been achieved by analysing the core cell-cycle engine (consisting of cyclins, CDKs and APC) and its regulation by external stimuli. The discovery of the autonomous, self-perpetuating component

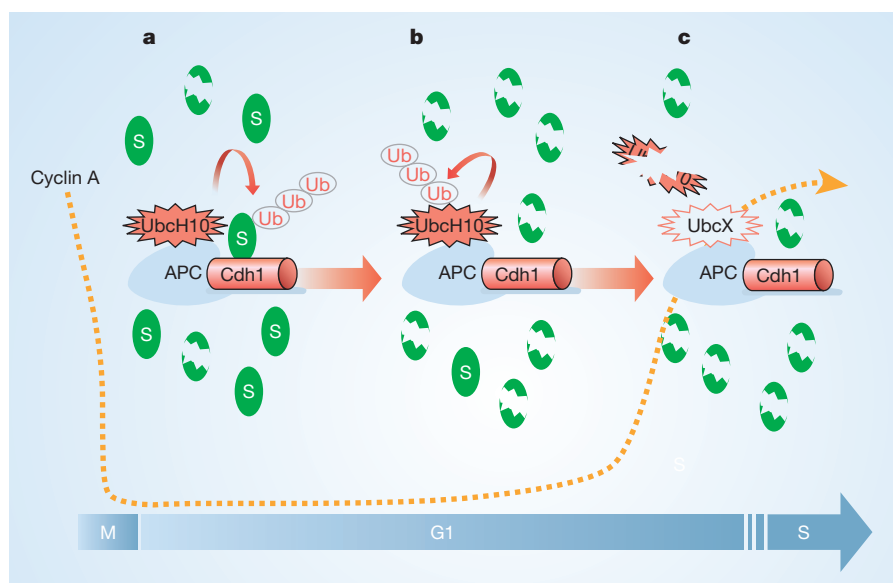


Figure 2 A mystery solved. Cyclin A accumulates towards the end of G1 phase, but its destroyer, APC, is still active. How can this happen? Rape and Kirschner¹ have provided the answer. They found that, in early G1 phase (a), UbcH10 is busy helping APC to find and add ubiquitin to substrates that remain from the preceding mitosis. b, Only after most of these have been degraded can UbcH10 add ubiquitins to itself, triggering its own destruction. c, The absence of UbcH10 allows cyclin A to accumulate, whereas other APC substrates can still be degraded through the actions of other Ub proteins (UbcX). The self-destruction of UbcH10 is an 'autonomous sensor' of mitotic completion, and also provides the molecular switch that allows cells to proceed from DNA segregation and cell division to the new round of DNA duplication.

of this fundamental mechanism¹ is an important addition to our understanding of the very basis of cellular multiplication. ■

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Cosmology

Neutrino probes of dark energy

Gia Dvali

Dark energy drives the accelerating expansion of the Universe — but what is dark energy? Its influence on the properties of neutrinos might be detectable, and could reveal something of its mysterious nature.

The discovery that the rate of expansion of the Universe is accelerating has created a confusing situation in cosmology and particle physics. Although the standard cosmological model has been confirmed by data from the WMAP space telescope and by other telescope surveys of the large-scale structure of the Universe, nobody knows why the cosmic expansion is accelerating. The effect has been attributed to some mysterious gravitational source called 'dark energy', but this name is merely a codeword for something unknown. Theoretical explanations have been postulated, some of which should be testable

through precise cosmological measurements and gravitational interactions. But now Kaplan *et al.*¹, in *Physical Review Letters*, and Fardon *et al.*², in the *Journal of Cosmology and Astroparticle Physics*, propose that innocuous subatomic particles — neutrinos — might offer a nongravitational means of probing the nature of dark energy.

The accelerating rate of expansion has been inferred directly from surveys of distant supernovae^{3,4}, but it also fits well with all other existing cosmological data. Supernovae, the cataclysmic explosions of dying stars, are often used in astronomical surveys as 'standard candles', because their brightness

is believed to be well understood. But very distant supernovae seem to be strikingly dimmer — and thus farther away — than expected. This suggests that, at large scales, the expansion of the Universe is accelerating, not slowing down; some mysterious repulsive force is making the Universe fly apart. The puzzling thing, however, is that this new force cannot possibly arise from either matter or radiation, because the gravitational force exerted by such sources could only make the expansion slow down (just as the Earth's gravity slows a stone thrown into the air).

Perhaps the accelerating expansion is due to the fact that gravity itself is modified at cosmologically large distances⁵. But if we assume that gravity remains 'normal' at reasonably large distances, then we are left with the option that the Universe must be filled with a mysterious source of gravity, whose properties are strikingly different from those of standard matter and radiation — dark energy. To generate a repulsive gravitational force, the new source must have an unusual 'equation of state' describing its behaviour, and must exert a large negative pressure. Also, as the Universe expands, the new source must be diluted at a much slower rate than any known form of matter or radiation.

What could this new, almost undilutable source be? Apart from modified gravity, the only such source considered until recently was the potential energy of a spatially uniform, scalar (Bose–Einstein) condensate, known as quintessence⁶. This energy acts in just the same way as Einstein's cosmological constant, inducing the accelerated expansion of space. In particular, this observation is the basis of so-called inflationary cosmology — the commonly accepted cosmological paradigm for the early history of our Universe that includes a period of exponential expansion, or inflation. Perhaps, 13 billion years after the Big Bang, we are once again entering an inflationary period driven by some scalar condensate.

The two new papers^{1,2} (the work of Fardon, Kaplan, Nelson and Weiner) present an interesting argument in which neutrinos are an integral component of the dark energy. They consider the existence of new particles (with masses of about 10^{-3} electronvolts) that generate the dark energy, and the possible interactions between these particles and the known particles of the standard model. As far as quarks and charged leptons (such as electrons) are concerned, there are severe constraints on the strengths of these interactions, called couplings. However, the authors point out that neutrinos — weakly interacting, very-low-mass particles — are exceptional, and cosmologically relevant couplings are still possible for them.

Indeed, if the dark energy consists of some very weakly coupled new species of particle, neutrinos offer a better way of probing it directly for a variety of reasons. Because

of their weak interactions, there is still much about neutrinos that we don't know (possible interactions with dark-energy particles included); and because the neutrino mass is so small, it is very sensitive to weak physics. Furthermore, neutrinos do not carry any conserved quantum numbers, such as electric charge or colour. Most particle interactions are allowed or forbidden on the basis of conservation of quantum number (for example, the total electric charge must be the same before and after an interaction). The only conserved internal quantum number that neutrinos might have is the so-called lepton number (the lepton number of the neutrino is 1, and that of the antineutrino is -1). But there are excellent theoretical reasons for thinking that the lepton number might not be conserved after all (in fact, this might be the reason for the matter–anti-matter imbalance in the Universe). If this is so, neutrinos are free to mix with the hidden particles responsible for the dark energy.

The authors show^{1,2} that, if the neutrinos do mix with a hidden sector of dark-energy particles, the masses of the neutrinos would respond to the finite density of relic neutrinos in the Universe, changing with the rate of expansion of the Universe. In this way, they have circumvented the standard argument against ordinary known particles being connected with dark energy: although the number density of the particles gets diluted as usual with the expansion, the energy density does not. Some fraction of the energy is stored in the masses of the particles, and some fraction in the scalar-field potential. The sum of the two can dilute very slowly, even though the number density of neutrinos dilutes quickly. In such a way, the gas of neutrinos filling the Universe is promoted into an undilutable substance, dark energy.

Neutrino masses that change over time in this way, depending on their environment, have profound phenomenological consequences. As Kaplan *et al.*¹ point out, this would open up the possibility of probing dark energy through neutrino oscillations—the transformation of one type of neutrino into another that has now been observed experimentally⁷. Neutrino masses are usually considered to be constants and independent of the density of a surrounding medium. With this condition relaxed, one might expect interesting deviations from the standard oscillation picture, deviations that might shed new light on existing experimental discrepancies.

The consequences for experiments could be significant indeed. If the neutrino mass changes on cosmological time scales, it does not affect structure formation in a standard way. Thus, the neutrino mass measured today might be larger than the values excluded for constant-mass neutrinos. From the ever more precise data on neutrinos from the Sun, backed up by data from the KamLAND experiment, which uses neutrinos generated

in nuclear reactors, definite predictions for the energy spectrum of solar neutrinos at the Earth can be made. Deviations from these predictions would signify new physics, such as the new scalar forces in this dark-energy model^{1,2}. With many neutrino experiments currently under way, there is an exciting possibility that terrestrial particle-physics experiments could soon shed light on the physics controlling the grandest scales of the Universe. ■

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Cell biology

Clathrin's Achilles' ankle

Frances M. Brodsky

The protein clathrin forms lattice-like coats on transport vesicles that bud from cell membranes. High-resolution models of the lattice reveal interactions involved in its disassembly once the vesicles have formed.

Cells are busy places, with membrane traffic moving in all directions—from the cell surface to internal compartments and back again, and from one compartment to another. The transport vehicles are tiny, membrane-bound spheres called vesicles, which bud off from the membrane that encloses a compartment (or the cell itself), and fuse with a target membrane. Cargo is sorted into vesicles by so-called coat proteins, of which one of the best known is clathrin.

Clathrin proteins assemble to form a lattice-like membrane coat, and a striking new high-resolution model of this coat is described by Fotin *et al.*¹ on page 573 of this issue. The model reveals the most detailed picture so far of clathrin's contacts in the lattice. On page 649 Fotin *et al.*² provide a further model, that of fragments of another protein, auxilin, bound to the lattice. From this, they suggest how auxilin could disrupt a lattice contact to trigger coat disassembly—an essential process for recycling clathrin and delivering cargo to its destination.

Clathrin-coated vesicles serve numerous functions in the cell. They internalize nutrient receptors and signalling receptors, regulating a cell's sustenance and its stimulation by environmental factors. They also recapitulate membrane at neuronal synapses and sort out proteins in the secretory pathway (reviewed in ref. 3). Clathrin itself has a remarkable three-legged (triskelion) shape, which is formed by three 'heavy chains' and three 'light chains' (Fig. 1a). The self-assembly of clathrin triskelions into a regular lattice (see Fig. 2 on page 574; ref. 1) provides the organizing template that allows receptors to be sequestered into vesicles. Meanwhile, 'adaptor' molecules stimulate lattice formation at membranes and trap receptors in the vesicle coat, concentrating themselves and the receptors in the lattice. So, receptor

sorting depends on the regulation of clathrin self-assembly and disassembly; such pathways can be elucidated by defining the molecular interfaces in the clathrin lattice.

The first structural model⁴ of the clathrin lattice (at 21 Å resolution) was generated six years ago, using electron cryomicroscopy and image averaging, and revealed that each lattice edge comprises leg segments from four different triskelions. The centre of a triskelion lies at each vertex; the legs extend outwards, spanning two edges and converging beneath another vertex. Fotin *et al.*¹ have now created a model at much higher resolution (7.9–11 Å resolution) by averaging the images of individual leg segments, taking advantage of their greater symmetry compared with that of the whole triskelion.

Clathrin has in fact been so well studied that only one unexpected—albeit very informative—feature emerges from the new model. But the model also provides invaluable information about how the known clathrin features fit into the bigger picture of the lattice. The familiar features include the fact that the entire linear portion of the triskelion leg, from the trimerization domain (TXD) to the terminal domain (TD), is formed by tandemly repeated structural motifs called clathrin heavy-chain repeats (CHCRs), previously observed in the heavy-chain X-ray structure⁵. The link between the far (distal) leg segment and the TD, previously thought to be flexible⁶, comprises the eighth CHCR and forms part of a newly defined ankle segment. The portion of the light chain that binds to the heavy chain localizes with the contacts previously identified from mutational analyses and a molecular-dynamics simulation, and places the central regions of the light chains with respect to the assembled lattice, confirming that they lie on the outside and parallel to the heavy chains⁷. The localization of heavy-chain sequences at

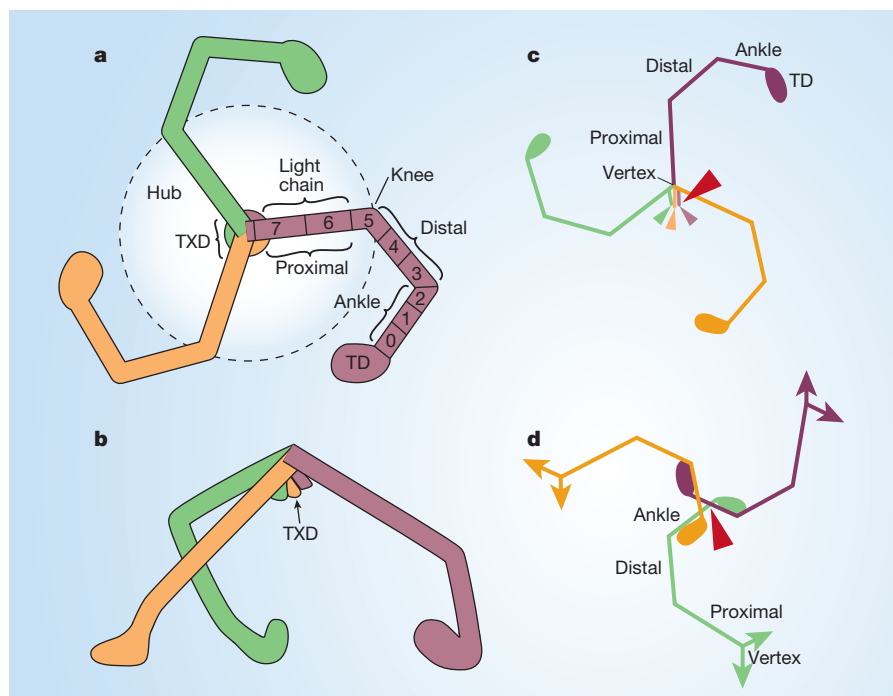


Figure 1 Views of the clathrin 'triskelion'. a, The triskelion is composed of three heavy chains (pictured) and three light chains (not shown). Each heavy chain comprises eight repeated motifs (CHCR 0–7), which make up the proximal, knee, distal and ankle segments of a clathrin leg, as positioned in the new model of the clathrin lattice¹. The region that interacts with the central third of a clathrin light chain is indicated. The trimerization domain (TXD), which extends beneath the vertex, starts with a helical hairpin at the carboxy terminus of CHCR7 and comprises an α -helix from each heavy chain. The hub region comprises the carboxy-terminal third of the heavy chains, including the proximal leg segments and the TXD. The heavy-chain amino terminus folds into the terminal domain (TD) and is attached to CHCR0 by a helical linker. b, Side view of the triskelion, showing the newly discovered orientation of the TXD¹. c, The red arrowhead indicates the carboxy-terminal ends of the α -helices involved in trimerization. In the lattice, this site is just above two crossed ankles of triskelia that pass underneath the vertex from two vertices away (see d). d, The red arrowhead indicates where two ankles cross underneath a vertex and where a bound auxilin fragment interacts. The legs from three triskelia are shown. It is proposed² that auxilin disrupts the lattice by interfering with the contacts between the sites indicated by arrowheads in c and d. The orientation of the TDs in d is intended to reveal the ankle crossings and does not necessarily indicate their orientation in the lattice.

the lattice vertices correlates completely with truncation experiments mapping the TXD^{8,9}.

It is the orientation of the TXD that is the most surprising feature of the new model¹. In the assembled lattice, the TXD invaginates into the centre, like an inward-poking belly-button (Fig. 1b), as opposed to outward-poking as was previously imagined. This orientation places the TXD next to the ankles of two further triskelia, centred two vertices away (Fig. 1c, d), revealing a previously unknown interaction in the lattice that might contribute to holding it together.

The ankle is considered to be the weakest leg joint (as personal experience confirms), and its vulnerability in the clathrin lattice is highlighted by Fotin and colleagues' second model², at 12 Å resolution, of an auxilin fragment bound to the lattice near two crossing ankles. Auxilin is a protein that helps to initiate lattice disassembly¹⁰ and hence vesicle uncoating; it does so by guiding a protein called Hsc70 to interact with the lattice. Hsc70 in turn hydrolyses adenosine triphosphate (ATP) molecules, producing adeno-

sine diphosphate (ADP) and the energy for disassembly^{11,12}. Understanding exactly where auxilin binds to the lattice is essential for unravelling the mystery of how ATP hydrolysis by Hsc70 triggers disassembly — a mystery that was first raised by a lower-resolution cryomicroscopy study¹³ that also localized auxilin to the lattice interior.

In Fotin and colleagues' models², the relative orientation of the ankles differs in the auxilin-bound and auxilin-free structures. From these data, and after fitting the NMR structure¹⁴ to their model, Fotin *et al.* propose a specific mechanism by which auxilin disrupts clathrin lattices. In this mechanism, an Hsc70 molecule that bound to a particular face of auxilin¹⁴ would lie between the TXD and its contact with the crossing ankles (Fig. 1c, d). Hydrolysis of ATP by Hsc70, and Hsc70's binding to a stretch of amino acids (peptide) in the clathrin molecule¹², might disrupt the TXD–ankle interactions and destabilize the lattice. That peptide could, the authors speculate, be part of the carboxy terminus of the heavy chain next to the TXD.

Additionally, the amino termini of the light chains might extend to the lattice interior and provide an Hsc70-binding peptide¹⁵. Although light chains are not strictly required for auxilin to disassemble lattices¹⁰, they have been implicated in this process¹⁶ and might contribute to the efficiency of uncoating *in vivo*.

In fact, a remaining mystery about clathrin concerns the light chains. What is the orientation of their regulatory domains, which flank the central heavy-chain-binding domain? This remains uncertain, although the fact that these regulatory regions could not be resolved in the new models hints at some degree of flexibility, indicating that they could potentially interact with each other, as suggested^{7,8}.

Through further analysis of these models^{1,2}, we can look forward to defining other contacts that mediate lattice assembly. Although the TXD–ankle interaction is a suggested target of disassembly, it is probably only one of many interactions that also contribute to assembly — clathrin assembles through many weak contacts between leg segments¹⁷ and adaptor molecules¹⁸. In fact, the central 'hub' fragment (Fig. 1a) of the clathrin triskelion can self-assemble, and antibodies against the hub disrupt clathrin lattices³. After modelling the leg orientation in two differently sized lattices, Fotin *et al.*¹ suggest that leg interfaces can rotate relative to each other using variable binding contacts. With the new models as a starting point for mutational studies, the positive features that control assembly — like the vulnerable ones that allow disassembly — should emerge. ■

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Cancer

Non-malignant clues to follicular lymphoma

N. Engl. J. Med. **351**, 2159–2169 (2004)

Non-Hodgkin's lymphoma is a group of cancers that affect white blood cells, and the course of disease is notoriously unpredictable. In some cases the cancer attacks the body aggressively; in others it advances more slowly, over several years. So choosing the best treatment for an individual patient remains in part guesswork.

Sandeep S. Dave and colleagues have addressed this problem in the case of follicular lymphoma, a subset of cancers that constitutes about a quarter of non-Hodgkin's lymphomas. They looked at the gene-expression signatures in 191 biopsy samples from patients suffering from untreated follicular lymphoma; on constructing a 'survival predictor' from the data, they found that certain gene activity could be associated with either a good or a poor prognosis.

Unexpectedly, say the authors, the expression signatures that predicted longer patient survival were derived from non-malignant immune cells that had infiltrated the tumours. The results provide further indications of the interplay between a person's immune system and renegade cancer cells, as well as prospects for a diagnostic tool for follicular lymphoma.

Roxanne Khamsi

Organometallic chemistry

Materials from flat carbon

J. Am. Chem. Soc. **126**, 15309–15315 (2004)

Since the work of van't Hoff and Le Bel in the nineteenth century, the fundamental geometry of the carbon atom has been recognized as tetrahedral: the four bonds around a fully saturated carbon atom point more or less towards the corners of a tetrahedron. In diamond, this tetrahedral coordination creates an extended, three-dimensional network of bonds. But, following 30 years of theoretical speculation, it has been suggested that tetracoordinate carbon might, in at least one special circumstance, have instead a planar geometry: two three-membered carbon rings joined end-to-end could form a stable C_5^{2-} ion, shaped like a flat bow-tie.

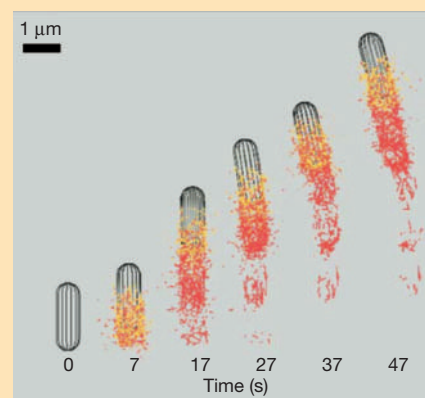
This molecule — stable in theory if coordinated to metal ions — has not yet been synthesized. But now Pattath D. Pancharatna *et al.* propose that it could be a building block for polymeric networks in one, two and three dimensions. Their quantum-chemical calculations suggest, for example, that bridging metal ions could link the bow-tie molecules into chains, or could

Cell biology

Bacteria step out *in silico*

PLoS Biol. **2**, e412 (2004)

Listeria monocytogenes has a bad reputation — it causes food poisoning. But this bacterium is a favourite of cell biologists because it offers so much information



about motility driven by the protein actin.

Listeria subverts a host cell's actin system to generate a ram force, produced by an actin 'comet tail', that drives the bacterium within and between cells. Jonathan B. Alberts and Garrett M. Odell have brought together an array of 80 computer processors, their own software and details from a large body of experimental data to model *Listeria* movement. Their aim was to go beyond previous simulations by taking account of more (and more complicated) interactions, both biochemical and mechanical.

This image is a simulation of the molecular processes that produce actin polymerization and motion, and shows one fruit of Alberts and Odell's labours. But the particularly notable feature of their results is the stepwise, nanometre-scale motion of the virtual bacterium, which is an emergent property of the model and is in line with observations. Refined models will help to clarify that behaviour, say the authors. They also hope that their approach will be useful in tackling complex subcellular systems in general.

Tim Lincoln

join them corner-to-corner in sheets. Zinc or lithium would work for the former, platinum for the latter. Zinc ions could also connect the C_5^{2-} units into three-dimensional networks containing zeolite-like channels — tempting targets indeed for the synthetic chemist.

Phillip Ball

Particle physics

Oscillating neutrinos on tap

Preprint at <http://arxiv.org/abs/hep-ex/0411038> (2004)

Neutrinos interact only very weakly with matter, which has made it tricky for scientists to find out more about them. Previous experiments have shown that neutrinos can spontaneously flip between three different 'flavours' (electron, muon and tau). Under the standard model of particle physics, this neutrino oscillation is only possible if they have mass.

E. Aliu *et al.* now report results from 'K2K', the first experiment to study oscillating neutrinos deliberately generated in a particle accelerator. A beam of muon neutrinos is generated at KEK, the National Laboratory for High Energy Physics in Tsukuba, Japan. A small neutrino detector checks the particles just 300 metres from the source, before the neutrinos race a further 250 km to the vast Super-Kamiokande neutrino detector at the Kamioka Observatory.

The team saw only 107 muon neutrinos out of the 151 expected at Super-Kamiokande if the neutrinos did not oscillate. This confirms the results of experiments that studied neutrinos coming through our atmosphere from space. The missing particles are thought to have switched flavour to become tau neutrinos.

Having proved that K2K works well, the authors will now press home the advantage of having a controllable, on-demand source of neutrinos to work out how and why they oscillate.

Mark Peplow

Biological techniques

Light engineering

Proc. Natl Acad. Sci. USA
doi:10.1073/pnas.0407645101 (2004)

The phytochromes found in plants, algae and bacteria are made up of a protein combined with a light-absorbing pigment. When the pigment soaks up light, the associated protein switches from one conformation to another and triggers a change in the cell.

Amanda J. Fischer and J. Clark Lagarias have converted a light-absorbing phytochrome into one that emits light. They introduced random mutations into a bacterial phytochrome and found one that, when illuminated, glowed bright red. The mutation concerned swapped one amino acid (tyrosine) for another (histidine).

Fischer and Lagarias propose that the key tyrosine normally lies in contact with the pigment and, when it absorbs light, it transmits this energy into the conformation change in the protein. The mutated amino acid is unable to do this, so the energy is instead re-released as red light.

Mutant phytochromes could find a use in cellular imaging experiments, the authors say, if they were genetically spliced to a second protein that researchers want to study. The engineered protein could be imaged at a particular time by adding a pigment of any colour, to bind the protein and trigger light emission.

Helen Pearson

Argentinian unhatched pterosaur fossil

New pterosaur-egg features add to our understanding of these extinct flying reptiles.

Our knowledge of the eggs and embryos of pterosaurs, the Mesozoic flying reptiles, is sparse. Until now, the recent discovery of an ornithocheirid embryo from 121-million-year-old rocks in China¹ constituted the only reliable evidence of an unhatched pterosaur. Here we describe an embryonic fossil of a different pterosaur from the Early Cretaceous lacustrine deposits of Loma del Pterodaustro (the Lagarcito Formation, which is about 100 million years old) in central Argentina. This new fossil provides insight into the eggshell morphology, early growth and nesting environments of pterosaurs.

The specimen (MHIN-UNSL-GEO-V 246; see Fig. 1 and supplementary information) is split into two slabs, with its articulated bones preserved as an aggregate (22 × 60 mm) in an oval shape. The porous appearance of the bone surface, the minimal ossification of the ends of the limb bones, and the absence of fusion between the sacral vertebrae and between the scapula and coracoid provide size-independent support of the specimen's early ontogenetic age^{2,3}. The skeleton is also preserved in the curled position typical of embryos⁴ — forelimbs folded against the body, hindlimbs flexed and skull facing caudally, rostrum tucked under a wing — as opposed to the spread-out configuration seen in most preserved pterosaur skeletons, including those of early juveniles³.

The proportionally large skull and the long forelimbs, with their robust metacarpals IV carrying elongated phalanges, identifies the specimen as a pterosaur⁵. Within the pterosaur group, the metacarpal:humerus ratio of greater than 0.8, the subequal lengths of the femur and metacarpal IV, and the proximally placed, D-shaped deltopectoral crest of the humerus, support the specimen's placement among the pterodactyloids^{6,7}. Pterosaurian fossils collected so far at Loma del Pterodaustro belong to the filter-feeding pterodactyloid *Pterodaustro guinazui*⁸. This specimen is indistinguishable from both juvenile and mature specimens of *Pterodaustro* — with its long, slender snout and evidence of filament-like mandibular dentition⁸.

The relative sizes of limb elements of the specimen, which give an estimated wingspan of 27 cm, are similar to those of early juvenile specimens of *Pterodaustro* and agree well with allometric observations based on a growth series of this taxon indicating negative and positive allometry for the proximal (humerus and ulna) and distal (metacarpal IV) portions of the wing, respectively⁹. The correspondence between the wing proportion of the

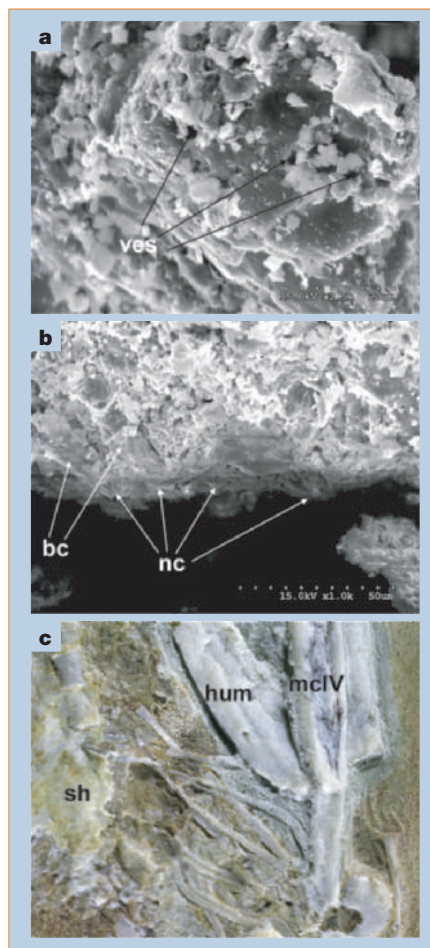


Figure 1 Shell around a pterosaurian embryo from the Early Cretaceous period in Argentina (specimen MHIN-UNSL-GEO-V 246). **a, b**, Scanning electron micrographs of radial sections of the eggshell: note the nucleation centres and bladed crystals at the apex of the V-shaped eggshell units, and micrometre-sized vesicles (ghost protein) on the surfaces. **c**, Detail showing the eggshell and general preservation of the bones. Abbreviations: bc, bladed crystals; hum, humerus; mcIV, metacarpal IV; nc, eggshell nucleation centres; sh, eggshell; ves, vesicles. For composite drawing of the entire specimen, see supplementary information.

specimen and those of previously published juveniles of *Pterodaustro*⁹ indicates a near-hatching stage of the former and a hatching stage of the latter, and suggests that neonatal growth rates were minimal.

A smooth, carbonatic material of biotic origin covers portions of the specimen and displays features of an eggshell, indicating that the new fossil is contained within an elongated egg (Fig. 1). Although the eggshell is somewhat weathered, it is extremely thin — about 30 μm in thickness. Scanning electron microscopy reveals that it consists of a single layer of calcite, with crystals radiating from a basal point and forming V-shaped eggshell units, similar to the eggshell of

archosaurs (including birds), whose units radiate from an organic core¹⁰. Preserved crystals near the nucleation centres also exhibit the blade shape that is characteristic of most archosaurian lineages.

No information on egg morphology was recovered with the previously reported pterosaurian embryo from China¹. Our specimen, however, shows that pterosaurs shared with extant archosaurs and chelonians an eggshell composed of a single layer of juxtaposed carbonatic crystals¹¹, but they differed from chelonians in that they laid calcitic eggs with blade-shaped crystals¹². The new fossil shows that pterosaurian eggs also differ from the hard-shelled eggs of squamates (geckos in particular), which lack nucleation cores and V-shaped eggshell units¹².

At Loma del Pterodaustro, individuals of *P. guinazui* vary greatly in size — wingspans range between 27 and 300 cm. Such a range, together with the presence of embryonic and neonate remains⁹, suggests that a nesting ground was nearby. Palaeoenvironmental reconstructions, combined with the remarkably low diversity of Loma del Pterodaustro — fish are the only vertebrates other than the hundreds of *Pterodaustro* fossils — suggest that this pterosaur inhabited an environment unsuitable for most other tetrapods, in agreement with reconstructions of this filter-feeding pterosaur as an ecological analogue of the flamingo. Furthermore, the association of the specimen with remains of early juveniles, subadults and adults of *Pterodaustro*^{8,9} agrees with previous inferences of parental care in pterosaurs¹³.

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Palaeontology

Pterosaur egg with a leathery shell

The recent discovery of a pterosaur egg with embryonic skeleton and soft tissues from the Yixian Formation confirmed that the flying pterosaurs were oviparous¹. Here we describe another pterosaur egg whose exquisite preservation indicates that the shell structure was soft and leathery.

The new pterosaur egg with its embryo (JZMP-03-03-2; Fig. 1 and supplementary information) was collected from the Jingangshan Beds of the Yixian Formation in the Jingangshan area of Liaoning, China. Both this and the earlier pterosaur egg (IVPP V13758) are from beds of Early Cretaceous age, estimated to be 121 million years old by ⁴⁰Ar/³⁹Ar dating².

The presence of the pteroid bone and the long wing-phalanges and wing-metacarpals within the egg shows that JZMP-03-03-2 is an embryonic pterosaur^{3,4} (for composite drawing, see supplementary information). The ilium and tibia are completely preserved, but the pubis, pteroid and dorsal vertebrae are only partially ossified. The pteroid has a slightly bent proximal end. The forelimb bones have embryonic characteristics, such as longitudinal shallow grooves on the shaft. The humerus is robust and complete, with a length of 17 mm; the deltopectoral crest is only weakly ossified. The radius and ulna (about 26 mm long) are straight and much longer than the humerus.

The right- and left-wing metacarpals are both well developed and are 13.3 mm long — about 53% of the ulna length. There are only three wing phalanges, as in the pterodactyloid *Beipiaopterus* from the Yixian Formation⁵. The robust first wing phalanx has a length of 15.8 mm, which is slightly longer than that of the wing metacarpal. The second wing phalanx narrows distally, and at 22.7 mm is much longer than the first; the third wing phalanx is 26.8 mm. The ratio of the combined length of wing phalanges to humerus is 3.84, which is lower than the ratio of 5.08 found in the adult specimen of *Beipiaopterus*. The difference in wing-finger length to humerus between JZMP-03-03-2 and the adult *Beipiaopterus* is consistent

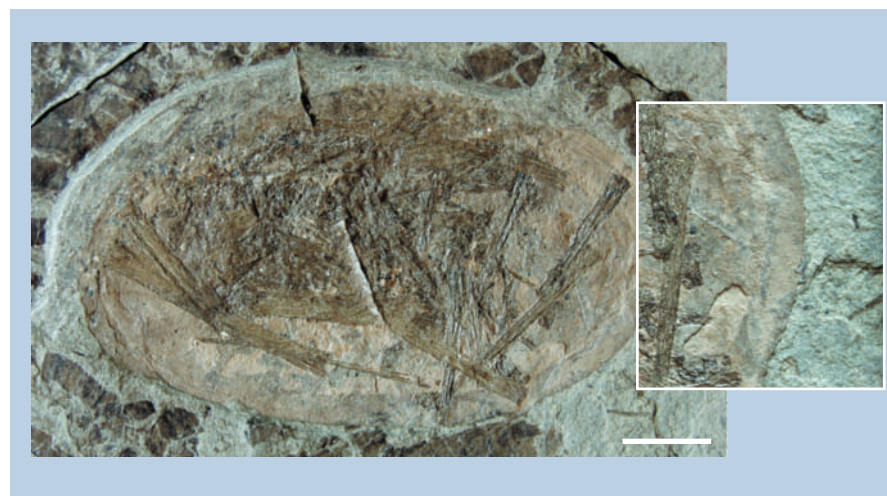


Figure 1 Early Cretaceous pterosaur egg and embryo (JZMP-03-03-2) from the Yixian Formation of Liaoning, China. Scale bar, 1 cm. Inset, magnification of egg boundary (130%) showing the thin, soft shell and no evidence of lamination structures. For composite drawing of the specimen, see supplementary information.

with the growth pattern of other pterosaurs⁶.

This specimen seems to be longer and narrower than the previously reported egg¹ (63.7 mm and 36.4 mm maximum length and width, respectively). Most skull elements (except four curved and needle-like teeth) are poorly ossified. Because of differences in some characters of the JZMP-03-03-2 and IVPP V13758 embryos (such as their shape and size for developmental stage), we suggest that the two eggs contain embryonic skeletons that belong to different pterosaur taxa.

The matrix in the interior of the egg is yellowish-brown; the embryonic skeleton is greyish-brown. The thin shell is brown-to-dark-brown and contrasts with the matrix surrounding the eggshell. The eggshell appears to be very thin (about 0.25 mm; Fig. 1, inset) and has no lamination in its microstructure. We did not detect the inner mammillary layer or the outer squamous layer formed of calcite crystallites that form the standard structural components of the hard eggshells of dinosaurs^{7,8} and of some modern amniotes⁹. Neither the part nor the counterpart of this pterosaur eggshell show any of the sharp fractures that are typical of broken, hard eggshells.

Because calcareous shells of molluscs are present in the same shale, we can rule out the possibility that the absence of a calcareous shell in this specimen might have been caused by diagenetic dissolution post mortem. Our observations indicate that this pterosaur egg from Yixian had a soft, leathery shell, similar to those widely found among sphenodonts, squamates, crocodiles and turtles⁹.

This pterosaur egg specimen is preserved in grey lacustrine tuffaceous shales with horizontal bedding, together with typical freshwater fossils such as fish, turtles, conchostracans, insect larvae, ostracods, gastropods, plants and lizards. Both sedimentary and palaeontological evidence indicates that the site of burial of these eggs was a low-energy

palaeoenvironment. But the shallow lake, to which these eggs were transported after death, is not the original nest site, which was likely to have been a lake beach or mud-flat.

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brief communications arising online

♦ www.nature.com/bca

Atmospheric science: Tropospheric temperature series from satellites

S. Tett & P. Thorne (doi:10.1038/nature03208)

Atmospheric science: Stratospheric cooling and the troposphere

N. P. Gillett, B. D. Santer & A. J. Weaver (doi:10.1038/nature03209)

Reply: Q. Fu, D. J. Seidel, C. M. Johanson & S. G. Warren (doi:10.1038/nature03210)

Atmospheric science

Tropospheric temperature series from satellites

Arising from: Q. Fu *et al.* *Nature* **429**, 55–58 (2004)

There has been considerable debate about changes in the temperature of the troposphere¹ measured using the Microwave Sounding Unit (MSU) instrument^{2,3} or radiosondes^{4,5}. Fu *et al.*⁶ linearly combine time series from two MSU channels to estimate vertically integrated 850–300-hPa temperatures and claim consistency between surface and free-troposphere warming for one MSU record. We believe that their approach overfits the data, produces trends that overestimate warming and gives overly optimistic uncertainty estimates. There still remain large differences between observed tropospheric temperature trends and those simulated by a climate model.

Fu *et al.*⁶ linearly combine MSU channels 2 and 4 using coefficients estimated from linear regression on a single, monthly mean radiosonde data set to create an effective weighting function that minimizes the effect of the stratosphere. For this approach to be valid on all space and time scales, the structure of stratospheric temperature variability must be stationary; this is not the case in reality. For example, the quasi-biennial oscillation⁷ has a temperature response of more than 1 K above pressures of 100 hPa, where the weightings of Fu *et al.*⁶ are negative, but little signal below 100 hPa, where the weightings are positive. Fu *et al.*⁶ will therefore alias an inverse quasi-biennial oscillation signal into the tropical tropospheric record — something not apparent in radiosonde observations.

Fu *et al.* trained and tested both their channel-2 and -4 coefficients on the same radiosonde data⁵, which can give false agreement and overfitting⁸. They found a global-average trend difference between

their estimated value and actual 850–300-hPa temperatures ($T_{850-300}$) of 0.001 K per decade. We believe that this result is misleading: their statistical model could have been independently confirmed by at least one other vertically resolved radiosonde data set⁴, a reanalysis⁹, a climate model forced with observed sea surface temperatures and anthropogenic and natural forcings¹⁰ or a coupled climate model forced with anthropogenic and natural forcings¹¹. We did this for tropical trends (Table 1). Discrepancies between tropical trends computed using the method of Fu *et al.*⁶ (T_{fjws}) and tropical $T_{850-300}$ trends range from –0.02 to 0.06 K per decade, with root-mean-square values ranging from 0.03 to 0.09 K. Except for HadRT2.1s, the T_{2IT} trend (where T_{2IT} is a synthetic channel for lower-middle troposphere) is a better estimate of the $T_{850-300}$ trends than T_{fjws} trends. These T_{fjws} trends are generally larger than the $T_{850-300}$ trends, suggesting that the approach of Fu *et al.* has a warm bias.

Trend discrepancies, and root-mean-square values, are smaller when T_{fjws} is compared with 1,000–100-hPa temperatures ($T_{1,000-100}$), with less evidence of systematic bias (Table 1). This is probably because of the form of the effective weighting function. However, there still exist differences of –0.01 to 0.02 K per decade between $T_{1,000-100}$ and T_{fjws} trends — about 10% of the observed surface tropical warming.

Average tropospheric temperature trends derived from an ensemble of coupled atmosphere–ocean model simulations are similar to those in the atmosphere-only case (Table 1). However, tropospheric trend

ranges in the coupled simulations are larger than the atmosphere-only case and so are consistent with that estimated from one processing of the MSU record³. This demonstrates that ignoring observed changes in sea surface temperature leads to a weaker test of model–data consistency.

We re-estimated the channel-2 and -4 coefficients of Fu *et al.*⁶ using HadRT2.1s (ref. 4), rather than the radiosonde data set of ref. 5. Our coefficients differ from those of Fu *et al.* and are sensitive to the choice of training period, with a total uncertainty of the order of 10% for global and tropical coefficients, corresponding to a trend uncertainty of 0.01 to 0.02 K per decade.

Although the approach of Fu *et al.* is novel, independent data indicate that it contains significant uncertainty. HadAM3 and the GISS model¹² forced with observed sea surface temperatures and forcing reconstructions show significantly greater warming than all ‘observed’ data sets. To resolve differences between models and observations requires good experimental design and process-based studies using physical understanding.

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Reply: Fu *et al.* reply to this communication (doi:10.1038/nature03210).

Table 1 Tropical trends for deep-layer temperatures for 1 December 1978 to 1 December 2002

Data source	T_4	T_2	T_{fjws}	T_{2IT}	$T_{850-300}$	$T_{1,000-100}$	Surface
MSU (Remote Sensing Systems ³)	–0.35	0.12	0.18				0.13
MSU (University of Alabama, Huntsville ²)	–0.39	0.05	0.10	0.00			0.13
Radiosonde (HadRT2.1s ⁴)	–0.60	–0.08	–0.02	0.03	0.00 (0.09)	–0.03 (0.07)	0.13
ERA40 reanalysis (1 Dec 1978–1 Dec 2001) ⁹	–0.20	0.06	0.09	0.02	0.03 (0.07)	0.10 (0.02)	0.10
HadAM3 ¹⁰ Model average	–0.29	0.19	0.25	0.20	0.22 (0.03)	0.23 (0.02)	0.14
Smallest trend	–0.31	0.17	0.22	0.18	0.21	0.21	0.12
Largest trend	–0.26	0.22	0.29	0.23	0.25	0.27	0.15
HadCM3 ¹¹ Model average	–0.45	0.16	0.23	0.21	0.21 (0.03)	0.22 (0.01)	0.16
Smallest trend	–0.42	0.07	0.13	0.12	0.12	0.13	0.10
Largest trend	–0.51	0.24	0.32	0.28	0.30	0.30	0.21

Tropical (30° S–30° N) trends (K per decade) are shown for deep-layer temperatures for 1 December 1978 to 1 December 2002. For the non-satellite data sets, static weighting functions were used to estimate synthetic Microwave Sounding Unit (MSU) equivalents. T_{fjws} is derived for each data set by applying the Fu *et al.* published coefficients to the T_2 and T_4 data. All data were zonally averaged, then cosine-weighted and least-square estimates of the linear trends computed from annual-mean data. For HadRT2.1s, Indian data were removed from the analysis. Also shown are the logarithms of the pressure-weighted 850–300-hPa temperatures ($T_{850-300}$) and of the pressure-weighted 1,000–100-hPa temperatures ($T_{1,000-100}$); the root-mean-square of the annual-mean differences between those and T_{fjws} is shown in brackets. Surface trends are from data averaged over land and ocean. For ERA40, we used two-metre temperatures over land and sea surface temperatures over the oceans. Surface temperatures from HadCRUT2v are used for RSS, UAH and HadRT2.1s. For the two model ensembles, the average, largest and smallest trends are shown. The difference between largest and smallest gives an indication of uncertainty in the ensemble average. The coupled (HadCM3) and atmosphere-only (HadAM3) simulations differ in their forcings, with the main differences being a correction of an error in ozone loss and changes to the sulphur cycle in the HadAM3 simulations. The HadAM3 (HadCM3) ensemble consists of six (four) simulations.

Atmospheric science

Stratospheric cooling and the troposphere

Satellite observations of tropospheric temperatures seem to show less warming than surface temperatures, contrary to physical predictions¹. Fu *et al.*² show that statistical correction for the effect of stratospheric cooling brings the satellite-based

estimates of tropospheric warming into closer agreement with observations of surface warming. Here we apply the method of Fu *et al.*² to output from a state-of-the-art coupled climate model and show that simulated tropospheric temperature trends are consistent with those observed and that their method is robust.

Tropospheric temperatures (T_2) monitored by channel 2 of the satellite-based Microwave Sounding Unit (MSU) include a contribution from temperatures in the cooling stratosphere^{1,2} (T_4). Using radiosonde data, Fu *et al.*² apply a regression method to quantify the relative influences of T_4 and T_2 on the mean temperature of the 850–300-hPa layer ($T_{850-300}$). We apply the Fu *et al.* regression method to simulated 1958–97 monthly-mean global-mean T_4 , T_2 and $T_{850-300}$ from a four-member ensemble of a climate-change experiment that was performed with the National Center for Atmospheric Research Parallel Climate Model (PCM)^{3,4} with combined anthropogenic and natural forcing.

The model-derived regression coefficients of $T_{850-300}$ against T_2 and T_4 are $a_2 = 1.106 \pm 0.026$ and $a_4 = -0.157 \pm 0.019$, with 5–95% uncertainty ranges derived from intra-ensemble variability. Results are in close agreement with the coefficients estimated by Fu *et al.*² from radiosonde data ($a_2 = 1.156$, $a_4 = -0.153$). To assess the contribution to these coefficients of the overlap between the T_2 and T_4 weighting functions, we derive a weighting function for $T_{850-300}$ and regress this directly against the mass-based weighting functions⁵ of T_2 and T_4 . This yields $a_2 = 1.089$ and $a_4 = -0.129$, implying that the regression relation derived by Fu *et al.* arises largely from the overlap of the weighting functions, rather than from physical coupling between tropospheric and stratospheric temperatures.

Because we know the actual $T_{850-300}$ trends over 1979–99 in PCM, we can evaluate the reliability of the statistical method of Fu *et al.*² for reconstructing these trends. For each ensemble member, trend reconstructions were produced with the Fu *et al.* and PCM-derived regression coefficients. Reconstructed trends agree with the actual PCM $T_{850-300}$ trends to within 0.016 K per decade on average (Fig. 1). We find a similar level of agreement between PCM's reconstructed and actual $T_{850-300}$ trends for the Northern and Southern Hemispheres and the tropics. Note that although simulated trends in T_{2IT} (where T_{2IT} is a synthetic channel for lower-middle troposphere) and $T_{850-300}$ are in close correspondence, PCM's T_{2IT} trends are not subject to problems that affect the observed T_{2IT} product, such as changes in surface emissivity, intersatellite calibration biases, and noise amplification².

Our model-based $T_{850-300}$ trends shown in Fig. 1 are consistent with the free tropospheric

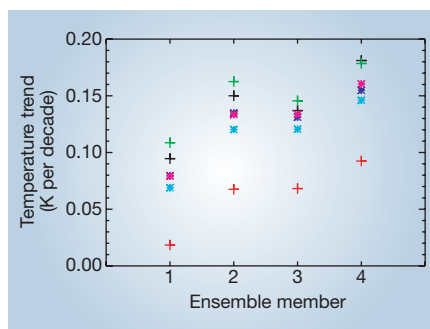


Figure 1 Simulated trends in global-mean free-tropospheric temperature. Black crosses, trends in $T_{850-300}$ over the period 1979–99, as simulated by the National Center for Atmospheric Research Parallel Climate Model (PCM) in each of four realizations of an experiment with anthropogenic and natural forcing^{3,4}. Asterisks indicate free-tropospheric temperature trends reconstructed from synthetic T_2 and T_4 trends using the method of Fu *et al.*². These are calculated using three different sets of regression coefficients, which are derived from radiosonde observations by Fu *et al.*² (pink asterisks), estimated from the PCM experiments (dark blue asterisks), and obtained directly from the T_2 and T_4 weighting functions (light blue asterisks). Red crosses, simulated trends in T_{2IT} ; green crosses, simulated trends in T_{2T} . The simulated trend in T_4 is -0.36 ± 0.03 K per decade. The model's surface warming over 1890–1999 (0.62 °C) is consistent with that observed.

pheric temperature trends that Fu *et al.* reconstructed from MSU observations for the period 1979–2001 (refs 2, 6; 0.09 and 0.18 K per decade for the UAH (University of Alabama at Huntsville) and RSS (Remote Sensing Systems) reconstructions, respectively). Overall, we find that the analysis method of Fu *et al.*² is robust, and that their radiosonde-based regression relationships between T_4 , T_2 and $T_{850-300}$ are in good agreement with those independently derived from climate-model output.

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Reply: Fu *et al.* reply to this communication (doi:10.1038/nature03210).

Fu *et al.* reply — The success of our method¹ for reconstructing tropospheric temperature trends is reinforced by Gillett *et al.*², who show that our method is robust for reconstructing the tropospheric temperature

trends, and that the statistical relationships between our T_4 , T_2 and $T_{850-300}$ estimates are in agreement with those independently derived from climate-model output. But Tett and Thorne³ use different data sets in the tropical region and suggest that our approach produces tropospheric temperature trends that are biased to warm and that it overfits the data. We argue that the differences in tropical tropospheric temperature trends between our estimate of $T(T_{fjws})$ and the $T_{850-300}$ of Tett and Thorne do not invalidate our method. We also question their interpretation of the comparison between global climate model results and satellite observations.

We first clarify that the quasi-biennial oscillation (QBO) issue that they raise is irrelevant to the trend analyses because the QBO signal is periodic. It is also incorrect that our weightings were negative above 100 hPa in the tropics. In reality, T_2 still has a significant weight above 100 hPa (ref. 4) and so should experience QBO effects. By contrast, our weighting function changes sign from positive to negative at about 75 hPa and has much smaller absolute values than the T_2 weighting function throughout the tropical stratosphere (see Fig. 3 of ref. 5).

Tett and Thorne show discrepancies between T_{fjws} and $T_{850-300}$ trends ranging from -0.02 to 0.06 K per decade for different data sets applied to the tropics, and so claim that our approach is biased to warm. We have shown that our weighting function is largely free of the stratospheric influence (Fig. 3 of ref. 5). However, these large trend differences arise because, in the tropical region, our weighting function is attributed to the whole troposphere from the surface to the tropopause (about 100 hPa), including a significant contribution from the layer between 300 hPa and 100 hPa, where the temperature trends are poorly characterized but might be very different from those below⁶.

For the tropical region, where the tropospheric temperature trends have significant height dependence, we argue that the T_{fjws} trend is more representative of the entire troposphere than of the $T_{850-300}$ layer. For example, for the ERA40 data for 1979–2001 in the tropics, there is a moderate positive temperature trend below 775 hPa, a moderate negative trend between 700 and 400 hPa, and a very strong positive trend between 300 hPa and tropopause. The $T_{850-300}$ trend excludes the large positive contribution from the upper troposphere, which leads to a much smaller trend for $T_{850-300}$ than for T_{fjws} . The large discrepancy with ERA40 data therefore arises owing to its very complex vertical structure of trends, which has not been shown to be realistic. If it is real, it indicates that the $T_{850-300}$ trend does not represent the layer-mean trend of the entire tropical troposphere but that the T_{fjws} trend does. The trend comparison, which shows a mean

difference of 0.01 K per decade between T_{fjws} and $T_{1,000-100}$ (mass-weighted temperatures from 1,000 to 100 hPa) in Table 1, supports our argument.

Tett and Thorne assert³ that the average tropospheric temperature trends from an ensemble of coupled atmospheric–ocean model simulations are similar to those in the atmosphere-only case. However, we notice that the average ratio of T_{fjws} to surface-temperature trends is about 1.4 from the coupled model, which is different from the atmospheric-only result (about 1.8); the former is consistent with the trend ratio derived from the RSS (Remote Sensing Systems) data (about 1.4) (Table 1). Understanding the differences between the HadAM and HadCM results are outside the scope of our study¹, but we notice that the global climate simulation with prescribed sea surface temperature does not conserve energy.

We performed tests, similar to those done by Tett and Thorne³, to check the sensitivity of the regression coefficients to different data sets⁶ and to the choice of training periods. The scatter in derived coefficients was within 10%, in agreement with their findings. Using the observed Microwave Sounding Unit T_2 and T_4 , this leads to an uncertainty of, at most, 0.01 K per decade in the derived tropospheric temperature trends. Our more recent analysis⁵, in which we directly apply our effective weighting function¹ to observed profiles of stratospheric temperature trend, reveals that our approach does remove the stratospheric influence effectively, leaving a residual influence of less than 0.01 K per decade⁵. Furthermore, by comparing T_2 and T_{fjws} in Table 1, we notice a robust stratospheric contamination in tropical T_2 trend, insofar as it depicts the tropospheric trend: it is about -0.06 K per decade

from both satellite Microwave Sounding Unit data sets, radiosonde, and both global climate model outputs (only ERA40 reanalysis shows a smaller stratospheric influence in T_2).

This reply was prepared with input from Kevin E. Trenberth.

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Molecular model for a complete clathrin lattice from electron cryomicroscopy

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Clathrin-coated vesicles are important vehicles of membrane traffic in cells. We report the structure of a clathrin lattice at subnanometre resolution, obtained from electron cryomicroscopy of coats assembled *in vitro*. We trace most of the 1,675-residue clathrin heavy chain by fitting known crystal structures of two segments, and homology models of the rest, into the electron microscopy density map. We also define the position of the central helical segment of the light chain. A helical tripod, the carboxy-terminal parts of three heavy chains, projects inward from the vertex of each three-legged clathrin triskelion, linking that vertex to 'ankles' of triskelions centred two vertices away. Analysis of coats with distinct diameters shows an invariant pattern of contacts in the neighbourhood of each vertex, with more variable interactions along the extended parts of the triskelion 'legs'. These invariant local interactions appear to stabilize the lattice, allowing assembly and uncoating to be controlled by events at a few specific sites.

Clathrin-coated vesicles carry lipids and proteins between intracellular, membrane-bound compartments^{1–3}. The coat is a lattice of clathrin, which surrounds a central membrane vesicle⁴. The clathrin assembly unit is a trimer of three very extended subunits, which radiate from a central hub^{5,6} (Fig. 1a). This triskelion comprises three ~190 kDa (1,675-residue) heavy chains, each bearing a single ~25 kDa light chain^{5,6}. A clathrin 'leg' is about 475 Å long⁷. Its characteristic curl as seen in electron micrographs allows us to distinguish a 'proximal segment', a 'knee', a 'distal segment', an 'ankle', a 'linker' and a 'terminal domain' (Fig. 1a). Except for the terminal domain, a globular element at the amino terminus of the heavy chain, the thickness of a leg is relatively uniform. The light chains associate with the heavy-chain proximal segments^{8,9}, through a central segment of 71 residues with a clearly recognizable, α -helical heptad repeat^{10,11}.

When triskelions assemble into a coat, the legs interdigitate to create a lattice of open hexagonal and pentagonal faces. The coats exhibit a range of designs⁴. Three of the smallest, built from 28, 36 and 60 triskelions, are illustrated in Fig. 1b; we refer to them respectively as the 'mini-coat', 'hexagonal barrel' and 'soccer ball'. The hexagonal barrel or the soccer ball is probably the smallest polyhedron that can accommodate a transport vesicle.

Clathrin recruits coated-vesicle cargo through intermediary proteins known as adaptors, which determine selective inclusion of membrane anchored proteins into coated vesicles and which form an interface between the outer clathrin coat and the incorporated, cargo-bearing, membrane bilayer^{1–3}. *In vitro* reconstitution of coats from purified clathrin and adaptor complexes provides relatively homogeneous specimens, with a large proportion of hexagonal barrels, suitable for study by electron cryomicroscopy^{12,13}. A reconstruction at 21 Å resolution¹⁴ showed that the legs of assembled clathrin triskelions interdigitate in the pattern illustrated in Fig. 2¹⁵. A triskelion hub lies at each vertex. The proximal segments radiate towards the three neighbouring vertices and project gently inward. The legs bend smoothly at the knees, and the distal segments extend towards the next vertex, where the ankles of three converging chains cross each other about 75 Å beneath the apex of the triskelion centred there. The terminal domains project towards the interior.

Two parts of the heavy chain are represented by crystal structures. The terminal domain (residues 1–330) is a seven-blade β -propeller, connected to an α -helical zigzag that forms the linker (residues 331–494)¹⁶. A substantial part of the proximal segment is likewise an α -helical zigzag¹⁷. The latter structure revealed a larger repeating motif of about 145 residues or five helical α -zigzags—the clathrin heavy-chain repeat (CHCR). Residues 543–1576 of the heavy chain

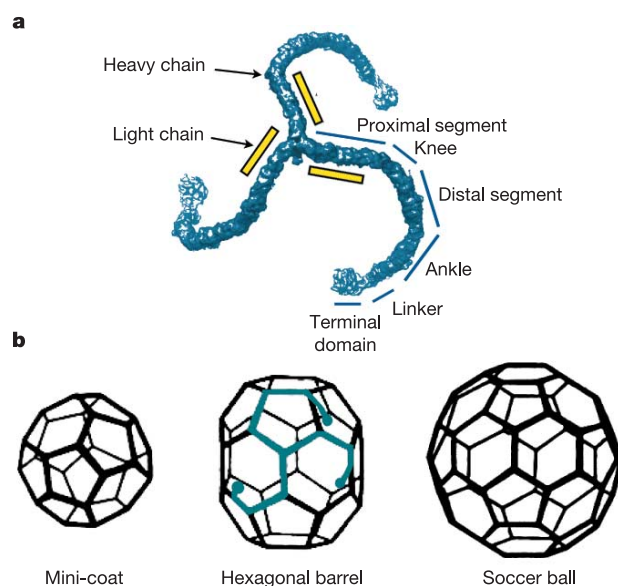


Figure 1 The clathrin triskelion and the designs of some simple clathrin lattices.

a, Clathrin triskelion, labelled with names for the segments of the heavy chain. The N terminus of the chain is in the terminal domain, and the C terminus is at the vertex. Positions of the light chains are shown schematically. **b**, Three structures that form when clathrin assembles into coats *in vitro*. Mini-coats have tetrahedral symmetry; hexagonal barrels, D₆ symmetry; soccer balls, icosahedral symmetry. Any surface lattice based on hexagons must have 12 pentagons to close completely. Schematic representation of one triskelion within the hexagonal barrel is shown in blue.

contain seven complete repeats, designated CHCR1–7 (ref. 17).

We have extended the resolution of clathrin hexagonal-barrel image reconstructions to subnanometre resolution (Fig. 2). We resolve a pattern of α -helical zigzags, along the complete leg, from the linker to the centre of the triskelion. The structure suggests answers to two questions that relate the molecular organization of the coat to its function in a cell. How does an assembling coat adapt to cargoes of different shapes and sizes? And how can the assembly of so elaborate a lattice be modulated by interaction with regulatory factors?

Image reconstruction of a hexagonal-barrel coat

We reconstituted clathrin coats *in vitro* from purified bovine-brain clathrin triskelions (either intact or light-chain free) and AP-2 adaptor protein complexes (Supplementary Fig. 1a). Presence or absence of light chains did not affect the distribution of lattice designs. We selected 1,450 particles from 225 electron micrographs of light-chain-free hexagonal barrels (Supplementary Fig. 1b, c); the D6-averaged image reconstruction had a nominal resolution of 12.5 Å (using the criterion¹⁸ that the Fourier shell correlation (FSC) = 0.143), a substantial improvement over the 21 Å resolution of the previous best structure¹⁴. The distribution of Euler angles for the particles (Supplementary Fig. 1d) showed that no orientations were missed. Each asymmetric unit of the barrel structure contains nine D6-symmetry independent clathrin legs¹⁵. By averaging corresponding segments of these legs, we could improve the signal-to-noise ratio and extend the resolution of the map. The nine independent legs align well in the proximal segment but diverge at the knee, where different degrees of bending propagate into significant changes in the positions of the distal segments and terminal domains relative to the triskelion vertex¹⁵. To exploit

local symmetry averaging, we split the clathrin leg into four segments, assumed to be invariant at the present resolution: proximal, distal, ankle and terminal-domain regions. We averaged the corresponding nine densities separately (see Methods). The densities of the knee regions were not averaged. The nominal resolution of the ‘non-coat symmetry’ (n.c.s.) averaged electron microscopy (EM) map was 7.9 Å for the proximal region (Supplementary Figs 1e and 2).

Comparison with an atomic model for residues 1280–1516 allows us to assess the reliability of the proximal-region cryoEM map (Fig. 3). The helices differ in their length and orientation, so that the model can be placed uniquely within suitably resolved densities. The averaged cryoEM density map indeed resolves individual helices in the proximal region, and we can match each helix of the atomic model with a corresponding rod-like feature of the cryoEM map (top portions in Fig. 3a–c). We can dock the atomic model as a rigid body into the cryoEM density with a correlation of 0.72. For comparison, we have computed electron density directly from the atomic model to a resolution of 7.9 Å (bottom portions in Fig. 3a–c); the calculated and observed densities are remarkably similar, confirming our assessment of the structural detail present in the EM map (see also Supplementary Fig. 2). After similar averaging of densities for the distal and ankle fragments, the nominal resolution estimates were 7.9 and 8.2 Å, respectively.

The X-ray structure of the terminal-domain-linker region¹⁶ was used as a model for local symmetry averaging of the terminal domain densities. Our placement of the terminal domain into the cryoEM density differs from the previous fit at 21 Å resolution¹⁵. Rotating the terminal domain atomic model by 180° around the linker axis gives much better agreement with density. The nominal resolution of this part of the map is 11 Å, and the density is weaker than for the rest of the triskelion. The relative disorder of the terminal domains is probably a consequence of both the lack of

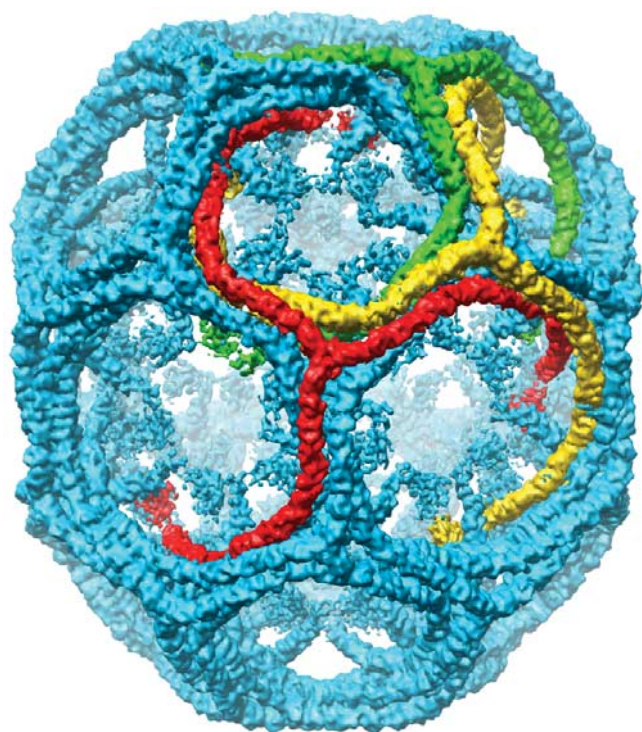


Figure 2 Image reconstruction of a clathrin hexagonal barrel (heavy chains only) at 7.9 Å resolution. There are 36 clathrin triskelions in the structure, which has D6 symmetry. Thus, there are three symmetry-independent triskelions (or nine symmetry-independent legs). The coloured triskelions show one choice of the three independent triskelions. Noisy central density, from spatially disordered and substoichiometric AP-2 complexes, has been flattened.

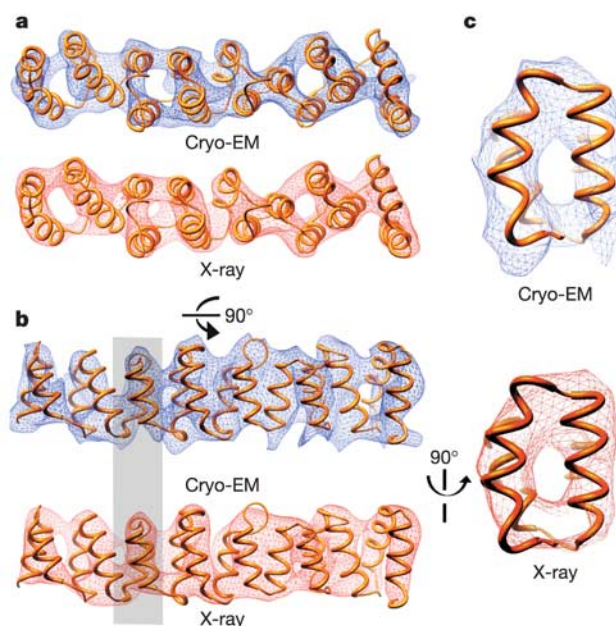


Figure 3 Rigid-body fit of the atomic model for a segment of the proximal leg¹⁷ to the density from the cryoEM image reconstruction. **a**, View from outside the barrel. In blue are contours of the cryoEM map; in red are contours of a map with a 7.9 Å resolution cut-off and a thermal parameter of 200, using structure factors calculated directly from the atomic model. **b**, Side view of the same. **c**, Cross-sectional view of the α -zigzag shown by the grey shading in **b**.

constraints from other contacts within the lattice and the asymmetric interactions some of them make with AP-2 complexes.

Molecular model for a clathrin triskelion

We used sequence alignment of CHCR fragments¹⁷ and homology modelling to derive atomic structures for the distal and ankle regions (Supplementary Fig. 3). In addition to CHCRs 1–7, we found that residues 395–542 within the linker conform well to the CHCR motif sequence profile. We designate them CHCR0.

We combined crystal structures and homology models to build atomic models for each segment of a clathrin leg. The averaged cryoEM density map had distinct structural details that allowed us to determine both translational and angular positions for each CHCR repeat within the map (Fig. 4). The docked CHCR models cover almost the entire length of a leg, without gaps or overlaps. The CHCR7 repeat extends into the trimerization region of a triskelion hub, and together with CHCR6 constitutes the proximal segment. CHCR5 forms the knee. The N terminus of CHCR5, as well as CHCRs 3 and 4, constitute the distal segment, and CHCRs 1 and 2 and the C-terminal part of CHCR0, the ankle. The helical hairpins rotate around the axis of their stack as they span a CHCR from N to C terminus, resulting in an intrinsic right-hand twist of helical

zigzags along a clathrin leg. CHCR6 in the proximal segment is rotated by roughly 90° about the leg axis relative to CHCR4 in the distal segment (Supplementary Fig. 4) and by about 180° relative to CHCR1 in the ankle (not shown).

Helical tripod

Each vertex in the unaveraged density map has a three-fold symmetric feature projecting inwards from the top of the hub. Averaging reveals a tripod of 50-Å-long, rod-like densities (Fig. 5). The rods extend from the vertex to the crossing of two clathrin ankles, which belong to triskelions centred two vertices away. We interpret these rods as α -helices. Between them and CHCR7 is an additional ‘proximal hairpin’ of density. We have built residues 1577–1597 into this density as a final α -zigzag, which starts at the C terminus of CHCR7, bends around, and continues into the helix of the tripod.

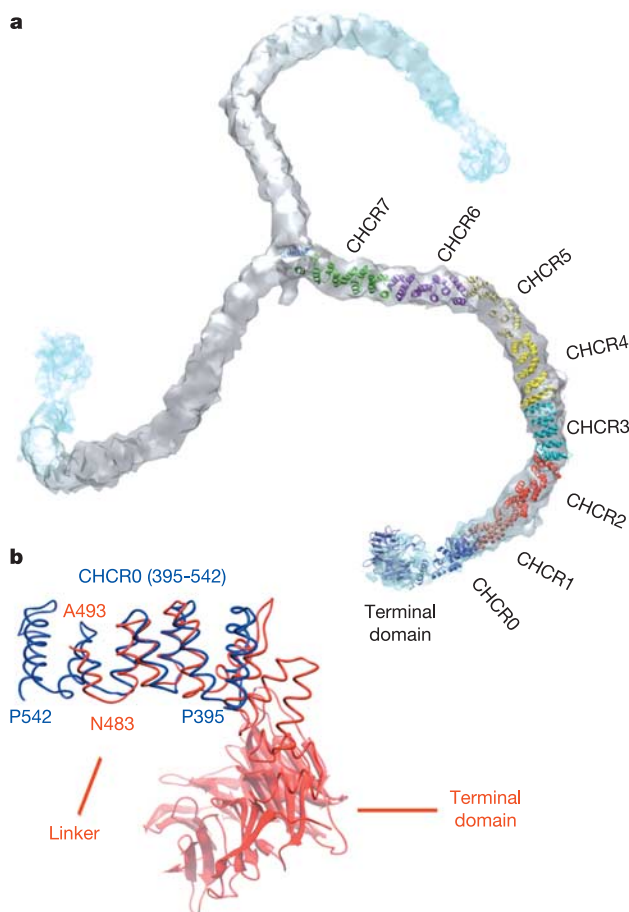


Figure 4 Backbone model for residues 1–1597 of the clathrin heavy chain. **a**, Fit into the clathrin density of the atomic model for terminal domain and linker¹⁶, the atomic model of CHCR6 and parts of CHCR5 and 7, and homology models of the rest of the heavy chain. For the colouring of CHCR segments and definitions of the residues at the boundaries, see Supplementary Fig. S3. **b**, Homology model for CHCR0 (blue) and the crystallographically derived model for the terminal domain and linker (red), superposed. The linker contains two additional helical hairpins, packed closely against the β -propeller terminal domain; the crystal structure lacks most of the final two hairpins of CHCR0.

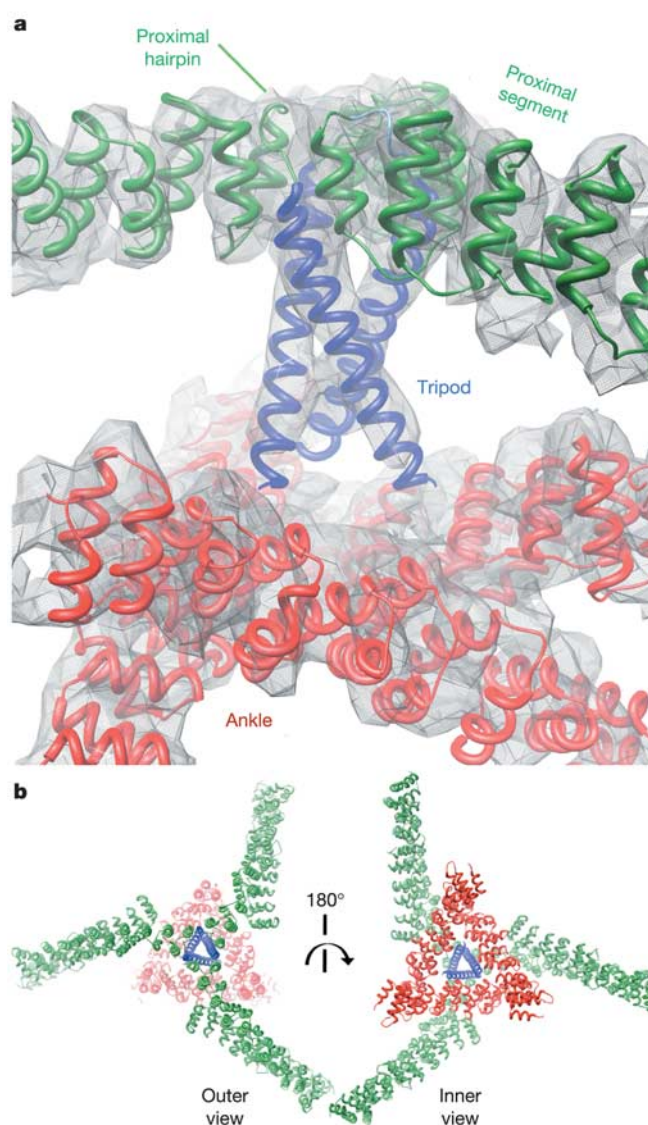


Figure 5 The hub assembly. **a**, Fit of the heavy-chain model into density surrounding one clathrin hub. Green, the C-terminal part of CHCR7 and the proximal hairpin. Blue, the tripod helix. Red, ankle segments from triskelions centred two vertices away. The colours are those of the corresponding CHCR segment in Fig. 4a. For simplicity, the density and model corresponding to knees are not included. **b**, Views of the same region (model only) from the outside (left) and inside (right) of the barrel. Note how the tripod links the apex of the hub assembly to the braided triangle of ankles that lies about 75 Å deeper within the structure (measured from the top of the triskelion vertex).

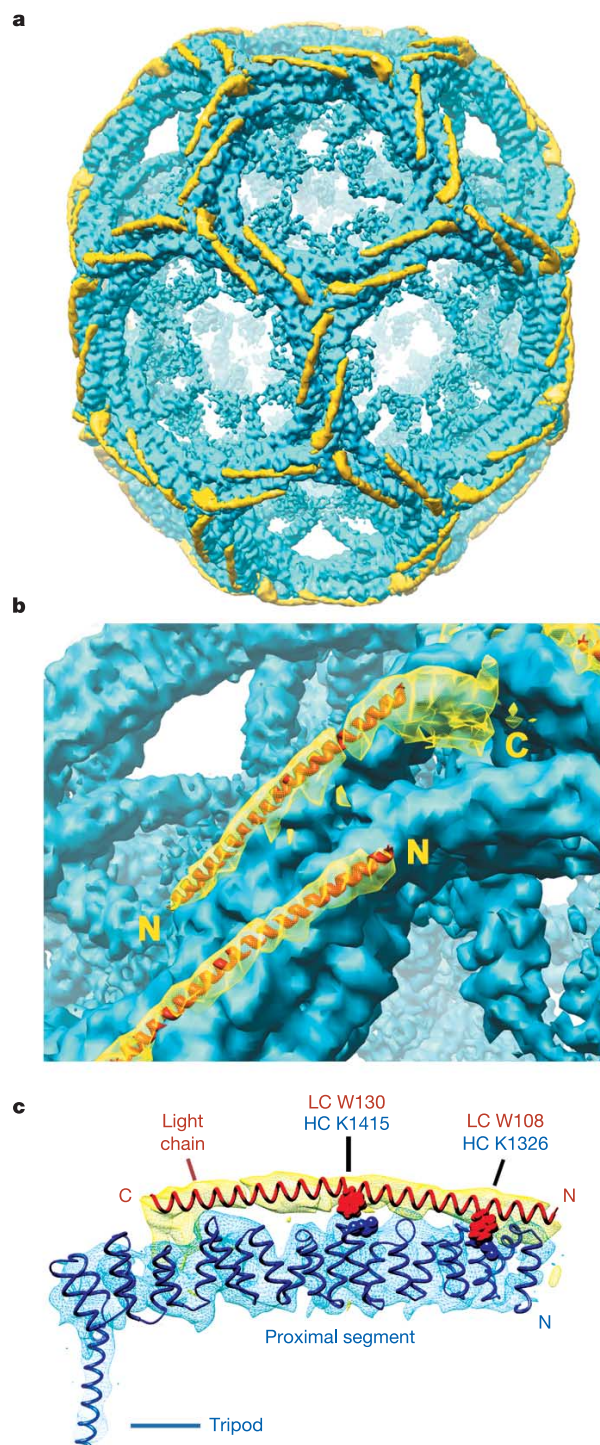


Figure 6 Model for clathrin light chains. **a**, Differences (yellow) between the image reconstructions with and without light chains, superimposed on the light-chain-free density (blue). **b**, Close-up of the vertex to the right of centre in **a**, with a helical model for the central 71 residues of the light chain positioned in the density. A globular, but somewhat diffuse, density for the C-terminal part of the light chain reaches around the hub of the triskelion. Much weaker density (not visible here) extends inward from the N-terminal end of the long helix, perhaps representing a disordered structure for the N-terminal part of the chain. **c**, View from the side of the light-chain model (red; map contours in yellow) in relation to the α -zigzags of the heavy chain (blue). Positions of two pairs of residues, shown by compensating mutations to interact with each other²³, are marked. LC, light chain; HC, heavy chain.

The proximal hairpins from three heavy chains stack against each other to form the top of the triskelion hub. A helical model for residues 1598–1630 fits well into the rod-like density of the tripod (Fig. 5). The orientation of the helix around its axis is not determined at the resolution of the map, but its sequence (Supplementary Fig. 5) suggests an amphipathic character and reveals a conserved pattern of repeating charged residues. Residues 1598–1630 are predicted to have high helical content; there are proline residues in one or another of the aligned sequences just at the boundaries of this segment, but none within it. Clathrin heavy-chain fragments truncated at residue 1615 trimerize properly, but shorter fragments do not¹⁹. The tripod helices diverge, and their C-terminal ends are not in contact, precisely consistent with the biochemical observations¹⁹.

The amino-acid sequence at the C terminus of a heavy chain (residues 1631–1675, extending beyond the tripod segment) is proline rich and varies among species more markedly than elsewhere in the clathrin heavy chain (Supplementary Fig. 5). Density potentially attributable to this final fragment merges with the ankle density, precluding definitive assignment. Our model thus includes 1,630 of the 1,675 residues in a clathrin heavy chain.

Invariant hub

There are three symmetry independent vertices in a D6-symmetric barrel, and as none of them corresponds to an actual three-fold axis of the coat, there are nine independent ways in which the various triskelions that interdigitate beneath each vertex could make contact¹⁵. We find, however, that the structures in the neighbourhood of each vertex are essentially three-fold symmetric (Fig. 5), and that the three different kinds of vertices can be superimposed (not shown). In other words, the pucker of the triskelion centred at each of the vertices, the contacts between the parallel proximal and distal segments in the adjacent edges, the splay of the helical tripods, and the contacts of the tripods with the ankles beneath them are all invariant. The knees vary in the sharpness of their bend¹⁵, but they curve gently enough to avoid the axis altogether, allowing the tripod to reach through to the ankles. The three ankle segments are braided into a triangular barrel (Fig. 5b), and the helices of the tripod terminate at the junction points of the ankle pairs, cross-linking the ankle triangle to the triskelion hub above them. The C terminus of the heavy chain may help stabilize interactions at the ankle joints, providing an ‘ankle brace’ to hold the entire structure together.

We describe by the phrase ‘invariant hub assembly’ (or, where clear from context, ‘invariant hub’) the tripod and three proximal segments radiating from a vertex, the distal segments (belonging to triskelions centred at nearest-neighbour vertices) lying just within those proximal segments (and running parallel to them) and the triangle of ankles (belonging to triskelions centred at second-nearest-neighbour vertices) at the foot of the tripod. We can think of the entire lattice as a set of such invariant hub assemblies, connected by more variably bent heavy-chain regions—the knees and the distal-segment-to-ankle junctions. The terminal domains and linkers hang inwards from the hub assemblies, to contact adaptors and a variety of accessory proteins.

The light chain

We selected 1,210 particles from 240 micrographs of clathrin coats containing both heavy and light chains, produced a reconstruction at 8.2 Å resolution, and made a direct comparison with light-chain-free coats by calculating a difference map (Fig. 6). The light-chain density divides into a 100-Å-long, central, rod-like feature; a less extended and more diffuse region at the end near the vertex of the lattice; and a third, very weak feature at the other end (Fig. 6b). The polarity of the extended structure is determined by early antibody localization studies, which showed that the C-terminal part of a light chain lies near a vertex^{9,20}.

The rod-like density corresponds to the central, α -helical, light-chain segment, as expected, because neither the N- nor the C-terminal region is required for incorporation into a coat²¹. The length of the rod-like density corresponds precisely to an extended 71-residue α -helix, ruling out an alternative, folded model²². Suppressor mutagenesis studies have pinpointed two contacts (HC K1326–LCa W108 and HC K1415–LCa W130, where HC and LCa indicate heavy chain and light chain a, respectively) in the heavy-chain/light-chain interface²³. We used this information to

dock a helical model into the cryoEM density map. When one of the two pairs of interacting residues is in contact, the second pair face each other as well (Fig. 6c). We can thus determine both the translational position of the helix along the rod of density and its angular orientation about the long axis. The light-chain helix interacts with a surface formed by interhelical loops in the heavy-chain proximal segment (Fig. 6c). The axis of the light chain, which tilts relative to the axis of the heavy chain, roughly follows the right-hand twist of the heavy-chain α -helical zigzags.

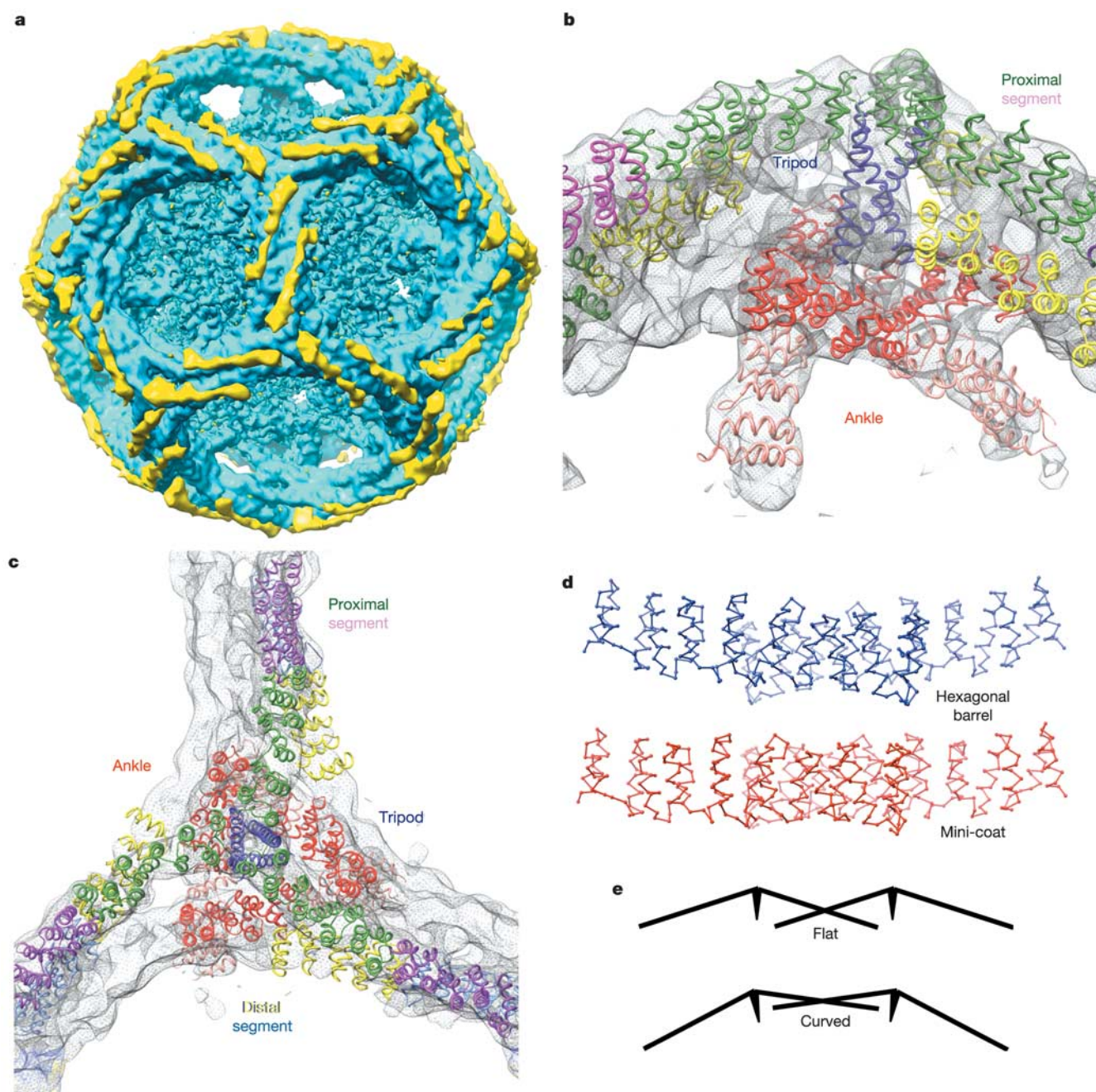


Figure 7 How clathrin forms lattices with different curvature: the mini-coat. **a**, Image reconstruction of a tetrahedral coat at 13 Å resolution. The central density, from disordered AP-2 complexes, has not been flattened in this illustration, unlike Fig. 2. The light-chain difference density is in yellow; the density from light-chain-free coats is in blue. **b**, The hub assembly region, showing the fit of the proximal–distal pairs (green and yellow, respectively), ankle segments (salmon and red) and tripod (blue), transplanted directly from the model of the hexagonal-barrel coat, without adjusting relative

orientations. Colours are those of the corresponding CHCR segment in Fig. 4a. **c**, A hub viewed from outside the coat, again with the directly transplanted model from the hexagonal barrel structure. Colour coding as in **b**. **d**, The crossing angles of two proximal segments in the edges of a hexagonal barrel (blue) and tetrahedral coat (red). The two differ by about 8°. **e**, Coats of different curvature can form from triskelions of constant pucker, by variation of the proximal–arm crossing angle. Projections of a pair of triskelions, with flat packing (upper) or curved packing (lower).

The functions of the clathrin light chains are still obscure. They appear to have no effect on assembly driven by adaptors (this work) or uncoating²⁴. The location of the light chain on the outside of the cage is consistent with a role in communicating with cytoplasmic structures rather than in cargo-oriented regulation.

Tetrahedral particles

Mini-coats were selected from the same micrographs used for the hexagonal-barrel image analysis just described. We used 920 particles of light-chain-free and 890 particles of light-chain-bound forms. Despite the small number of particles, the final tetrahedral symmetry averaged reconstructions (Fig. 7a) were reliably computed to resolutions of 13.2 Å for light-chain-free and 13.0 Å for light-chain-bound coats. There are seven symmetry-independent clathrin legs within a tetrahedral asymmetric unit, but we did not attempt further segmental averaging, as we did with D6 barrels. A mini-coat is about 620 Å in diameter, 80 Å smaller than a hexagonal barrel; its lattice is therefore more sharply curved. It nonetheless has the same overall organization and triskelion packing as seen in the hexagonal barrels (Fig. 7b, c). The light-chain map, computed as a difference between light-chain-bound and light-chain-free mini-coats (Fig. 7a), closely resembles the light-chain difference map for the barrels.

Lattice adaptability

Clathrin can form structures of variable curvature, ranging from the small mini-coats to extended hexagonal arrays^{4,25}. This adaptability is critical for function, as coated pits must engulf cargo of different shapes and sizes. Recent studies of clathrin dynamics illustrate that cargo diameter dictates the size (and hence the growth time) of a coated pit as it assembles at the plasma membrane²⁶. We can imagine two distinct molecular mechanisms for achieving variable curvature with a single, trimeric assembly unit. In one, the anti-parallel contacts within an edge of the lattice remain essentially invariant, while the pucker at a triskelion vertex changes to fit the required local curvature. In the other, heavy-chain contacts within an edge vary, leaving the pucker constant while altering the crossing angle between antiparallel proximal–distal pairs (Fig. 7e). We have compared mini-coat and hexagonal-barrel coats, which have different lattice curvatures. If we extract from a hexagonal-barrel coat the model of an invariant hub, it fits with virtually no change in pucker into the hub density of a mini-coat (Fig. 7b, c). In other words, the difference in the curvatures of the two lattices resides in the crossing angles between proximal–distal pairs along the edges (Fig. 7d), not in changes in the organization of a vertex.

The curvatures of mini-coat and hexagonal-barrel lattices are only moderately different, and both are sharper than the average endocytic coated vesicle. Thus, we cannot rule out the possibility that in formation of extended, flat arrays, such as have been seen on the ventral surface of cells in culture and on endosomes, there will be additional points of flexibility. Some of the mini-coat hubs that embrace both pentagonal and hexagonal faces do have slight but detectable deviations from three-fold symmetry. We suggest, however (based on the total of 16 symmetry-distinct interactions we see in the barrel and mini-coats), that conservation of the proximal–distal contacts (which link triskelions centred on neighbouring vertices) and of the ankle brace (which links second-nearest neighbours) will be true of all specific clathrin assemblies.

The invariant contacts just described are primarily polar in character and restricted in extent. They are confined to the inner levels of the heavy-chain imbrications—just where we expect regulation by membrane-associated proteins to occur. In the accompanying paper²⁷, we show that auxilin, an important regulator involved in uncoating, associates with the hub assembly, where it could recruit the uncoating ATPase, Hsc70, to destabilize the ankle-brace contacts.

Methods

Coat preparation, data collection and initial image processing

Coats were assembled²⁸, micrographs recorded, visually inspected on an optical diffractometer and only those showing clearly visible Thon rings²⁹ were digitized, particles picked, and initial reconstructions calculated as described in Supplementary Information. The first stages of image processing were performed using IMAGIC³⁰, resulting in a symmetry averaged three-dimensional reconstruction³¹.

Image refinement

Contrast transfer function (CTF) parameters were determined using CTFTILT³². Only micrographs for which CTF parameters could be determined accurately (fitting correlation coefficient >0.20) were selected. CTF parameters were calculated individually for each particle using values from CTFTILT (average defocus in two perpendicular directions, astigmatism angle at the micrograph centre, position of tilt axis and tilt angle) and *x* and *y* coordinates of the particle centre.

The three-dimensional model calculated from class averages was used as an initial reference for refinement by FREALIGN version 6.07 (ref. 33), which determines angular and shift parameters of the particles, corrects for CTF and computes a three-dimensional reconstruction. FREALIGN was run on a 30-node Linux cluster managed by LSF software (Platform Computing Corporation). Search and refinement of particle orientations were limited initially to a resolution range of 800–40 Å. A brute-force search (FREALIGN mode 3), with an angular step of 10°, was used to determine Euler angles and *x,y* shifts of individual particles relative to the initial model. This search was followed by 10 cycles of iterative refinement. At this stage all particles were included in the three-dimensional reconstruction.

In subsequent refinement, we used only particles showing high cross-correlation with the reference (FREALIGN PRES < 62°, from 800 Å to 40 Å). Particle images were first re-cut from the original micrographs averaged over 3 × 3 pixels (pixel size, 4.2 Å; Nyquist limit, 8.4 Å). After 30 iterations, refinement in the 800–8.4 Å resolution range converged, as judged by stable PRES values. Particle images were then again re-cut from the original micrographs averaged over 2 × 2 pixels (pixel size, 2.8 Å; Nyquist limit, 5.6 Å). The final 10 refinement cycles were run on a 64-bit AMD Opteron workstation and included data in the 800–5.6 Å resolution range. Density in the centre of the three-dimensional reference model used for refinement was masked out using a soft-edged mask with a 40-pixel radius for 3 × 3 binned images and a 60-pixel radius for 2 × 2 binned images. At the end of the refinement process, a tight mask was applied to the three-dimensional reference model to flatten density corresponding to the surrounding solvent (FREALIGN XSTD parameter set to 2.0).

The nominal resolution of the final reconstruction was estimated from the spatial frequency at which the FSC fell to 0.143 (ref. 18). Reconstructed maps were low-pass filtered to exclude data beyond this resolution. A negative B-factor of 1,000 Å² was applied (in reciprocal space, as $\exp(-0.25Bs^2)$; *s* is resolution in Å⁻¹) to the final reconstruction, to restore high-resolution contrast. The difference between light-chain-bound and light-chain-free coat densities was computed using a resolution bin-dependent scaling procedure implemented in EMAN³⁴. Chimera³⁵ and O³⁶ were used to produce surface-rendered views of density.

Local symmetry density averaging

Local symmetry EM density averaging used O³⁶ and MAVE³⁷ (Uppsala Software Factory). The atomic model of the proximal region¹⁷ was placed into each of the local symmetry-related positions. A transformation from the reference position to each symmetry-related position was found with LSQ_EXPLICIT in O, using least-square minimization of distances between pairs of residues 1279, 1327 and 1516 in reference and rotated models. A 7.5-Å-radius mask was created around the atomic models. MAVE, which optimizes the real-space cross-correlation between the masked reference and symmetry-related density (command IMPROVE), was used to refine the operators, which were then used for local symmetry density averaging and for projecting the average onto a mask in the reference position (command AVERAGE). The averaged density was expanded onto masks in the rotated positions (command EXPAND). Density overlap from the expansion was removed using MAMA³⁸. The FSC after n.c.s. averaging (Supplementary Fig. S1e) was computed by dividing the particles into two groups and calculating for each group an independent image reconstruction, which was then averaged (in segments) using the n.c.s. operators.

Modelling atomic structures of clathrin fragments

Three-dimensional atomic structures of CHCR0-5 and CHCR7 were modelled (using MODELLER^{39,40}) as described in the caption to Supplementary Fig. S3.

Fitting atomic models into cryoEM maps

Clathrin atomic models were inserted into the local symmetry averaged clathrin coat EM density map using O³⁷. The fit was optimized using rigid body refinement in MAVE (command IMPROVE). The reference electron density used for the optimization was calculated from the atomic model and filtered to the nominal resolution of the corresponding EM map using CCP4 programs SFALL and FFT⁴¹. Correlation coefficients of the fitting as output by MAVE ranged from 0.6 to 0.8. Positions of some of the terminal helices (helices *a*, *b* and *i*, *j*) in the models for CHCR0-5 and CHCR7 were shifted to provide a better fit to density. Helical structures of the tripod and the light-chain fragment were modelled using Insight II (Accelrys).

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A synaptic laminin–calcium channel interaction organizes active zones in motor nerve terminals

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Synapse formation requires the differentiation of a functional nerve terminal opposite a specialized postsynaptic membrane. Here, we show that laminin $\beta 2$, a component of the synaptic cleft at the neuromuscular junction, binds directly to calcium channels that are required for neurotransmitter release from motor nerve terminals. This interaction leads to clustering of channels, which in turn recruit other presynaptic components. Perturbation of this interaction *in vivo* results in disassembly of neurotransmitter release sites, resembling defects previously observed in an autoimmune neuromuscular disorder, Lambert–Eaton myasthenic syndrome. These results identify an extracellular ligand of the voltage-gated calcium channel as well as a new laminin receptor. They also suggest a model for the development of nerve terminals, and provide clues to the pathogenesis of a synaptic disease.

A principal aspect of synapse formation is the differentiation of an axonal segment into a nerve terminal specialized to release neurotransmitter after calcium influx. Although neurons grown in isolation can synthesize and release neurotransmitter, target-derived signals have a critical role in localizing presynaptic differentiation to sites of axon-target contact, thus ensuring precise apposition of the nerve terminal to the postsynaptic apparatus^{1,2}. Several putative target-derived organizers of presynaptic differentiation have now been characterized *in vitro*^{3–5}. Genetic studies in mice have demonstrated a critical role *in vivo* for one of them, laminin $\beta 2$ (refs 6–8).

Laminins are heterotrimeric ($\alpha/\beta/\gamma$) glycoproteins present in the basal laminae that coat the surfaces of many cell types. At least 15 trimers, assembled from five α -, three β - and three γ -subunits, have been isolated⁹. Skeletal muscles synthesize multiple laminins: ones containing the $\beta 1$ subunit predominate along most of the surface, whereas those containing the $\beta 2$ subunit are predominant in the small stretch of basal lamina that extends through the synaptic cleft at the skeletal neuromuscular junction (NMJ)¹⁰. $\beta 2$ -containing heterotrimers and $\beta 2$ fragments act as ‘stop signals’ for motor neurites and promote presynaptic differentiation *in vitro*^{11,12}; mice lacking $\beta 2$ laminin show marked defects in maturation of motor nerve terminals, leading to a lethal decrease in synaptic efficacy^{6–8}.

How does laminin $\beta 2$ exert these effects? Laminins bind to several classes of membrane proteins, including integrins and dystroglycan⁹, but these receptors do not seem to account for the observed distinction between $\beta 1$ and $\beta 2$ laminins. We therefore sought additional laminin $\beta 2$ receptors. We report here that laminin $\beta 2$ binds directly to the pore-forming (α or Ca_v) subunit of the voltage-gated calcium channels (VGCCs) that are concentrated in the presynaptic membrane and required for transmitter release. We show that this interaction accounts, at least in part, for the formation and maintenance of presynaptic specializations in motor nerve terminals.

Laminin and calcium channels interact

The starting point for this study was our previous finding that $\beta 2$ -containing laminins and VGCCs are associated in the Torpedo electric organ, a rich source of NMJ-like synapses. In that study, a

multimolecular complex precipitated by antibodies to the VGCCs contained laminin 9 (the $\alpha 4/\beta 2/\gamma 1$ heterotrimer)¹³. To ask whether laminin and VGCCs are nearest neighbours (immediately adjacent proteins) in this complex, we attempted to crosslink them. Synaptic membranes were treated with a cleavable, biotin-containing crosslinking agent¹⁴. The membranes were then solubilized with detergent and precipitated with antibodies to VGCCs. The crosslinker was cleaved, leaving biotin on both products. The two major proteins in the precipitate were laminin and the VGCCs, indicating that they are closely associated (Fig. 1a, lanes 1–6). No other major components were detected when the crosslinked complex was precipitated with antibody to VGCCs then denatured and reprecipitated with antibody to laminin, suggesting that laminin’s nearest neighbour (distance ≤ 2.3 nm) is the VGCC (Fig. 1a, lanes 7, 8).

This close association might reflect direct binding but might also arise if laminin and VGCCs are brought into close proximity as the synapse forms. To distinguish between these possibilities, we assayed the binding of beads coated with purified laminin 9 (Supplementary Fig. 1) to non-neuronal cells (human embryonic kidney 293 (HEK293) cells) that had been transfected with complementary DNAs encoding the pore-forming ($\text{Ca}_v 2.1$) and auxiliary ($\beta 1b$, and $\alpha 2\delta$) subunits of the rat VGCC. More VGCC-expressing cells bound laminin-coated beads than did adjacent, VGCC-negative cells, whereas few cells of either type bound control beads (Fig. 1b, c). Only a minority of transfected cells bound beads, because cell surface expression of VGCCs was low under these conditions: $\sim 50\%$ of cells bound beads in a stably transfected line (data not shown). Binding occurred at both 16°C and 37°C , was maximal by 30 min incubation, and was not markedly sensitive to divalent cations. Thus, laminin and the VGCC can interact rapidly and in a non-synaptic environment, suggesting that the interaction is direct.

The $\text{Ca}_v 2.1$ and $\alpha 2\delta$ subunits of the VGCC have extracellular domains whereas the $\beta 1$ subunit (required for surface expression of Ca_v) is entirely intracellular^{15,16}. Laminin-coated beads bound cells transfected with only $\text{Ca}_v 2.1$ and $\beta 1b$ subunits just as effectively as cells expressing $\text{Ca}_v 2.1$, $\beta 1b$ and $\alpha 2\delta$ (Fig. 1d). Thus, laminin interacts with the pore-forming subunit of the VGCC.

A set of ten genes encode pore-forming (Ca_v) subunits of the

VGCC¹⁶. To ask whether all Ca_v subunits are capable of interacting with laminin, we transfected cells with cDNAs encoding P/Q-, N-, R- and L-type channels (Ca_v2.1, Ca_v2.2, Ca_v2.3 and Ca_v1.2, respectively). P/Q- and N-type channels are mainly responsible for transmitter release from adult and developing motor nerve terminals, respectively, as well as from nerve terminals at central synapses^{17,18}. More laminin-coated beads bound to cells expressing Ca_v2.1 or Ca_v2.2 than to cells expressing Ca_v1.2 or Ca_v2.3 (Fig. 1e). Thus, laminin interacts preferentially with the major VGCCs of motor nerve terminals.

Binding sites on laminin

In the next series of experiments we defined site(s) on the laminin heterotrimer (Fig. 2a) that are required for its interaction with the VGCC. To ask whether the ability to bind VGCC is confined to Torpedo laminin 9 ($\alpha 4\beta 2\gamma 1$), we tested purified mammalian laminins. Beads coated with a mixture of laminins 10 and 11 ($\alpha 5\beta 1\gamma 1$ and $\alpha 5\beta 2\gamma 1$) bound to cells expressing Ca_v2.1 or Ca_v2.2 subunits, whereas beads coated with laminin 1 ($\alpha 1\beta 1\gamma 1$) did not. Neither laminin 1 nor laminin 10/11 bound to cells expressing Ca_v1.2 or Ca_v2.3 (Fig. 2b). The subunit that distinguished VGCC-interacting laminins from the non-VGCC-interacting laminin was $\beta 2$, which is concentrated in the synaptic cleft from an early stage of development¹⁰. We therefore tested a carboxy-terminal 20-kDa fragment of $\beta 2$ ($\beta 2$ -C) previously shown to be adhesive for motor neurons and to promote presynaptic differentiation^{11,12}. Beads coated with this fragment bound to cells expressing the Ca_v2.1 subunit but not to cells expressing Ca_v2.3 (Fig. 2c and data not shown). Within this fragment, we previously mapped a tripeptide—leucine-arginine-glutamate (LRE)—that is necessary for promoting adhesion of neurons and stopping neurites^{11,19}. A $\beta 2$ -C fragment in which LRE had been mutated to the inactive QRE did not detectably interact with VGCC-expressing cells, whereas an agonist decapeptide centred around LRE interacted with VGCC-expressing cells to the same extent as the

wild-type $\beta 2$ -C fragment (Fig. 2c). Similarly, soluble $\beta 2$ -C and multimerized decapeptide, but not the mutated fragment, inhibited interaction of laminin-9-coated beads with VGCC-expressing cells (Supplementary Fig. 2). Together, these results demonstrate that the LRE-containing site on laminin $\beta 2$ is necessary and sufficient for laminin interactions with the VGCC. The VGCC may be the 'LRE receptor' previously hypothesized to account for some effects of laminin on motor neurons^{11,19}.

Binding sites on the VGCC

To identify a laminin interaction site on the VGCC, we searched for sequences in extracellular domains that are conserved between Ca_v2.1 and Ca_v2.2 subunits (which interact well with laminin) but absent from Ca_v2.3 and Ca_v1.2 (which interact poorly). One region tested was the 11th extracellular loop (Fig. 3a), which is close to critical pore-forming residues and close to the site at which specific channel-blocking toxins bind²⁰. Ca_v2.1 is 78% identical to Ca_v2.2 in this region but only 56% and 39% identical to Ca_v2.3 and Ca_v1.2, respectively (Supplementary Fig. 3a). Antibodies generated to the Ca_v2.1 loop recognized extracellular epitopes on cells transfected with Ca_v2.1 or Ca_v2.2 but not Ca_v2.3 or Ca_v1.2 (Supplementary Fig. 3b). These antibodies blocked binding of laminin-coated beads to VGCC-transfected cells (Fig. 3b), suggesting that sites necessary for the VGCC–laminin interaction are near the 11th extracellular loop.

To investigate whether the critical sites are actually contained within the 11th extracellular loop, we generated chimaeric VGCC subunits in which this loop within the Ca_v1.2 and Ca_v2.1 subunits were exchanged, and tested their ability to bind beads coated with laminin 9 or the LRE-containing decapeptide. A molecule derived entirely from Ca_v1.2 except for the presence of a Ca_v2.1 loop supported adhesion to the same extent as wild-type Ca_v2.1, whereas the reverse chimaera (Ca_v2.1 subunit with the Ca_v1.2 loop) was unable to mediate adhesion (Fig. 3c). We also generated soluble recombinant peptides corresponding to 46 amino acids from the

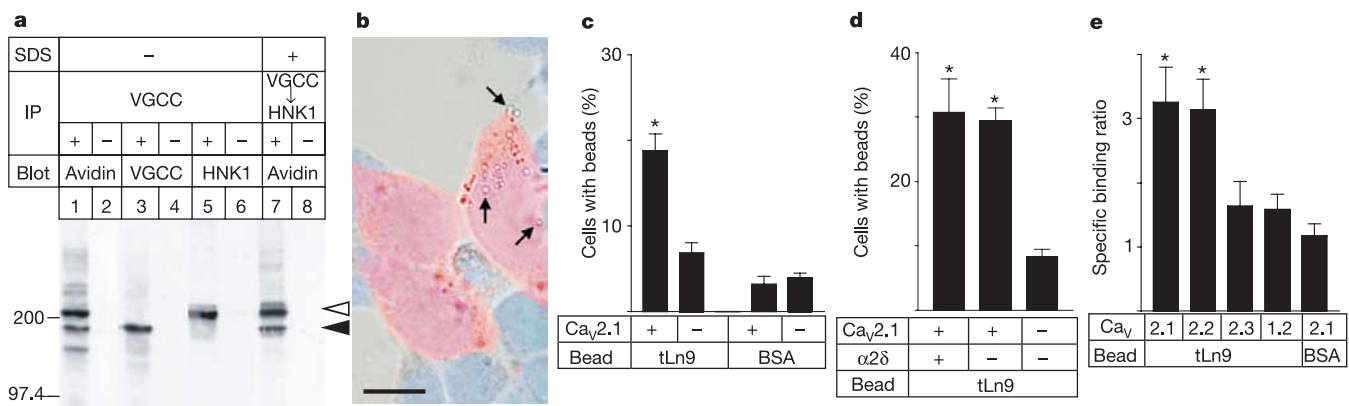


Figure 1 Laminin 9 interacts with pore-forming (Ca_v) subunits of calcium channels. **a**, Synaptosomes from Torpedo electric organ were crosslinked with a biotin-containing reagent; extracts were then immunoprecipitated (IP) with antibodies to VGCC. The crosslinker was cleaved, then proteins were separated on gels and blotted with antibody to VGCC or a carbohydrate epitope selectively associated with laminin 9 (H1NK1; ref. 13), or with avidin (lanes 1–6). Primary antibody was omitted in lanes marked '–'. VGCC and laminin are directly crosslinked. Analysis after immunoprecipitation with anti-VGCC, SDS-denaturation and re-precipitation with anti-H1NK1 (lanes 7 and 8) shows that these two proteins are each other's nearest neighbours in the complex. Filled and open arrowheads indicate VGCC and laminin, respectively. **b**, **c**, VGCC–laminin interaction assay. Beads coated with Torpedo laminin 9 (tLn9) were incubated with HEK293 cells that had been transfected with Ca_v2.1, $\beta 1b$ and $\alpha 2\delta$ VGCC subunits. A greater number of

VGCC-expressing cells (red; stained with anti-VGCC) bound beads (phase-bright dots; three are marked with arrows) than did neighbouring VGCC-negative cells (blue; stained with DAPI). Few cells of either type bound BSA-coated beads. Panel **b** shows a representative field whereas panel **c** shows quantification. Scale bar, 10 μ m. **d**, Binding of Torpedo laminin-9-coated beads to VGCC-transfected cells does not require the $\alpha 2\delta$ subunit. **e**, Torpedo laminin 9 bound to cells expressing the Ca_v2.1 (P/Q-type) or Ca_v2.2 (N-type) channels, but not to cells expressing the Ca_v2.3 (R-type) or Ca_v1.2 (L-type) channels. The 'specific binding ratio' is the percentage of VGCC-expressing cells with beads divided by the percentage of neighbouring VGCC-negative cells with beads. Each graph summarizes data from two to four separate experiments; asterisks indicate values that are different from the control at $P < 0.001$, Student's t -test. Graphs show mean $\pm$ s.e.m.

11th extracellular loops of $\text{Ca}_v1.2$ and $\text{Ca}_v2.1$, and asked whether they inhibited binding of laminin-9- or LRE-coated beads to VGCC-expressing cells. The $\text{Ca}_v2.1$ -derived peptide was inhibitory whereas the $\text{Ca}_v1.2$ -derived peptide was not (Fig. 3d).

Together, these results indicate that the LRE site on laminin $\beta 2$ binds to the 11th extracellular loop of the $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$ subunits. To test this idea directly, we generated recombinant proteins in which the $\beta 2$ -C fragment or its LRE $\rightarrow$ QRE mutant were fused to alkaline phosphatase²¹, and then used enzyme activity to monitor binding of the fragment to the $\text{Ca}_v2.1$ loop. In both solid-phase (Fig. 3e) and solution assays (Supplementary Fig. 4a) $\beta 2$ -C bound specifically to the $\text{Ca}_v2.1$ loop; however, little or no binding was seen for $\beta 2$ -C to the $\text{Ca}_v1.2$ loop, or for the LRE $\rightarrow$ QRE mutant to either loop. In both assays, the apparent dissociation constant (K_d) was ~ 250 nM (data not shown). Similarly, >10 -fold more laminin-9-coated beads bound to the $\text{Ca}_v2.1$ loop than to the $\text{Ca}_v1.2$ loop (Supplementary Fig. 4b). Thus, the LRE site on the laminin $\beta 2$ subunit and the 11th extracellular loop

of the VGCC Ca_v subunits appear to be major mediators of the binding between these two large multisubunit proteins.

Laminin $\beta 2$ -VGCC interaction induces vesicle clustering

Laminin $\beta 2$ is concentrated in the synaptic cleft at the NMJ from an early stage of synaptogenesis¹⁰, so one potential role of laminin $\beta 2$ -VGCC interactions could be to aggregate VGCCs in the presynaptic membrane. We used cultured motor neurons to test this possibility. Motor neurons purified from mouse spinal cord²² were cultured on laminin 1, which does not bind VGCC. After 2 days of neurite

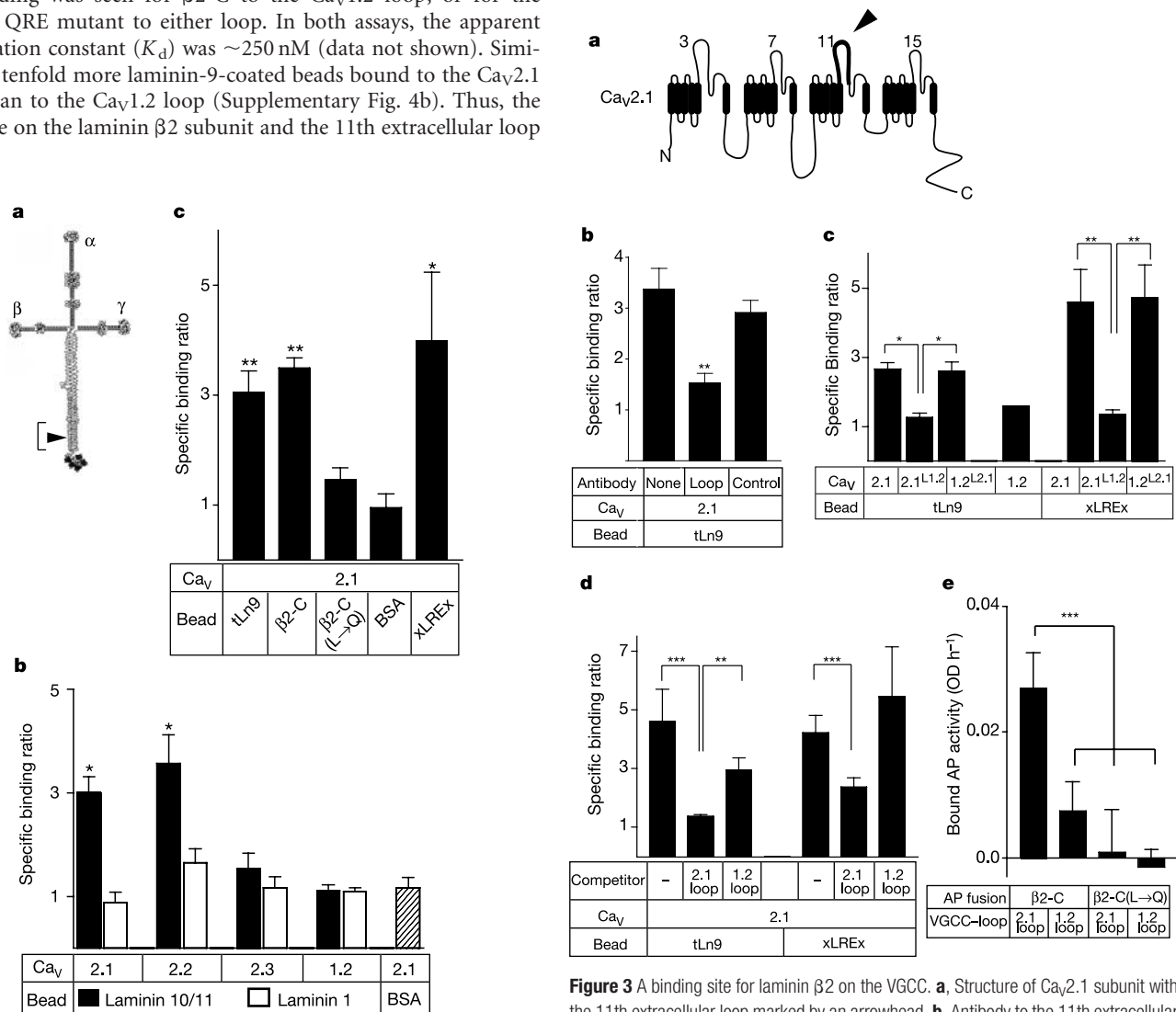


Figure 2 A binding site for VGCC on laminin $\beta 2$. **a**, Structure of laminins, which are cruciform heterotrimers of α -, β - and γ -subunits. The bracket and arrowhead indicate the $\beta 2$ -C fragment used in **c**, and the site of the LRE sequence within it, respectively. **b**, Beads coated with laminin 10/11 bind to cells expressing $\text{Ca}_v2.1$ or $\text{Ca}_v2.2$ but not $\text{Ca}_v2.3$ or $\text{Ca}_v1.2$. Laminin-1-coated beads show no specific binding. **c**, Beads coated with $\beta 2$ -containing Torpedo laminin 9, $\beta 2$ -C, or multimerized (8X) LRE-containing decapeptide¹¹ (xLREx) bind to $\text{Ca}_v2.1$ -expressing cells, but beads coated with control protein (BSA) or a mutant $\beta 2$ -C fragment (LRE $\rightarrow$ QRE, $\beta 2$ -C(L $\rightarrow$ Q)) do not. The specific binding ratio is calculated as in Fig. 1. Each graph summarizes data from two to four separate experiments; asterisks indicate values that differ from BSA-coated beads at $P < 0.0002$ (**b**) or $\beta 2$ -C(L $\rightarrow$ Q)-coated beads at $P < 0.01$ (**c**; single asterisk) or $P < 0.003$ (**c**; double asterisk), Students t -test. Graphs show mean $\pm$ s.e.m.

Figure 3 A binding site for laminin $\beta 2$ on the VGCC. **a**, Structure of $\text{Ca}_v2.1$ subunit with the 11th extracellular loop marked by an arrowhead. **b**, Antibody to the 11th extracellular loop of $\text{Ca}_v2.1$, but not control antibodies (anti-peroxidase or anti-IgG), inhibits binding of Torpedo laminin 9 to $\text{Ca}_v2.1$ -transfected cells. **c**, Binding of Torpedo laminin 9 and an LRE-containing decapeptide (xLREx) to chimeric calcium channels, demonstrating that critical residues for binding are on the $\text{Ca}_v2.1$ loop. 2.1^{L1,2}, $\text{Ca}_v2.1$ backbone with $\text{Ca}_v1.2$ loop; 1.2L2.1, $\text{Ca}_v1.2$ backbone with $\text{Ca}_v2.1$ loop. **d**, Binding of Torpedo laminin 9 and xLREx to $\text{Ca}_v2.1$ is inhibited by soluble $\text{Ca}_v2.1$ -derived loop but not $\text{Ca}_v1.2$ -derived loop (2 μM). **e**, Direct interaction of laminin $\beta 2$ - and VGCC-derived peptides measured by binding of $\beta 2$ -C-alkaline phosphatase or $\beta 2$ -C(L $\rightarrow$ Q)-alkaline phosphatase fusion proteins to $\text{Ca}_v1.2$ - or $\text{Ca}_v2.1$ -derived 11th extracellular loop. VGCC peptide was immobilized on nitrocellulose-coated plastic, which was then incubated with laminin fusion protein. After washing, retained alkaline phosphatase was assayed. Each graph summarizes data from two to three separate experiments. Brackets mark values that differ at $P < 0.001$ (single asterisk), $P < 0.01$ (double asterisk) or $P < 0.05$ (triple asterisk), Students t -test. OD, optical density. Graphs show mean $\pm$ s.e.m.

outgrowth, we added protein-coated beads, incubated the cultures for an additional 3 days, and then stained them with antibodies to the VGCC. VGCCs clustered at ~60% or ~75% of the sites at which growth cones were contacted by beads coated with laminin 9 or LRE-containing decapeptide, respectively, but at <15% of sites contacted by beads coated with control proteins (Fig. 4a, c and data not shown).

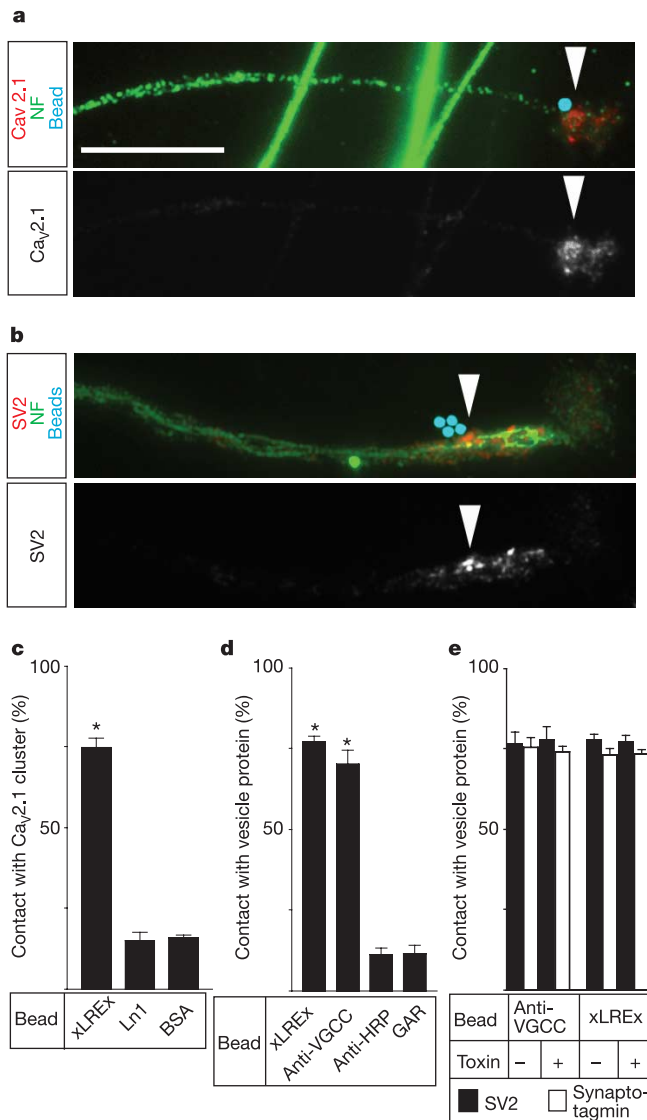


Figure 4 Laminin $\beta 2$ promotes presynaptic differentiation by clustering VGCCs. **a, b**, Beads coated with LRE-containing decapeptide (xLREx) were added to cultured motor neurons, which were subsequently stained for anti-Cav2.1 (red in **a**) or SV2 (red in **b**) and neurofilaments (green). Beads, photographed with phase optics, have been pseudocoloured blue. Cav2.1 and SV2 are concentrated at bead contact sites (arrowhead). The lower micrographs show only anti-Cav2.1 or SV2 immunoreactivity. Scale bar, 10 μ m. **c**, Quantification of VGCC clustering at bead contact sites as in **a**. Clustering was rare at sites of contact with control beads. Ln1, laminin 1. **d**, Quantification of synaptic vesicle clustering (SV2 immunoreactivity) at bead contact sites as in **b**. SV2-positive puncta were present at sites of contact with xLREx- or anti-VGCC-coated beads, but not control beads. GAR, goat anti-rabbit IgG. **e**, Experiments such as those in **b, d** were repeated in the presence or absence of agatoxin-IVA (100 nM) plus conotoxin-GVIA (3 μ M) to block calcium current through Cav2.1 and Cav2.2 channels. Blockade did not prevent vesicle clustering (assayed with antibodies to SV2 or synaptotagmin) at bead contact sites. Each graph summarizes data from two to three separate experiments; asterisks indicate values that differ from control at $P < 0.0001$, Student's t -test. Graphs show mean $\pm$ s.e.m.

What is the consequence of VGCC clustering? Staining with antibodies to a synaptic vesicle protein, SV2, showed that laminin-induced, VGCC-rich areas correspond to sites of vesicle aggregation (Fig. 4b, d). This result suggests that VGCC clustering leads to presynaptic differentiation, but this differentiation might also reflect a VGCC-independent effect of laminin $\beta 2$. To distinguish between these possibilities, we took two approaches. First, we clustered VGCCs directly using beads coated with anti-VGCC antibody, and asked whether other presynaptic components^{23,24} co-localized with the channels. Beads coated with anti-VGCC, but not with control antibodies, induced substantial presynaptic differentiation, as monitored by staining for integral components of synaptic vesicles (SV2 and synaptotagmin), a vesicle-associated peripheral membrane protein (synapsin) and a component of the active zone (bassoon) (Fig. 4d, e and data not shown). Second, we asked whether the 11th extracellular loop of Cav2.1, which selectively interferes with laminin-VGCC interactions (see above), prevented beads from clustering vesicles in neurites. The Cav2.1 loop blocked clustering, but the Cav2.2 loop did not (Supplementary Fig. 5). Thus, induction of presynaptic differentiation by laminin $\beta 2$ is mediated, at least in part, by the VGCC.

We next asked whether laminin-VGCC interaction promotes presynaptic differentiation by increasing calcium influx through the channel. Patch-clamp recordings from VGCC-transfected HEK293 cells failed to reveal significant effects of the LRE-containing decapeptide on channel properties (W. Yuan and J. Nerbonne, personal communication), but these non-neuronal cells might lack components necessary for modulation of channel function. We therefore asked whether the LRE-containing decapeptide or anti-VGCC were capable of clustering nerve terminal components in the presence of agatoxin-IVA plus conotoxin-GVIA, which prevent calcium flux through P/Q- and N-type VGCCs, respectively²⁵. These toxins had no effect on LRE- or anti-VGCC-induced vesicle aggregation (Fig. 4e), indicating that VGCCs need not be active in order to mediate presynaptic differentiation.

Light microscopic identification of active zones

Next, we wanted to investigate what role the laminin $\beta 2$ -VGCC interaction has *in vivo*. VGCCs are associated with release sites (active zones)²⁶, so we hypothesized that these interactions might affect the number or structure of active zones. So far, however, active zones at the NMJ have been visualized only by electron microscopy, which is cumbersome and poorly suited for assessing synaptic topology. We therefore sought a light microscopic stain for active zones. Bassoon, which is concentrated at active zones of central synapses, has been reported to be absent from NMJs²⁴, but we detected its expression immunohistochemically in cultured motor neurons and by *in situ* hybridization in adult motor neurons *in vivo* (data not shown), leading us to re-examine this issue by confocal microscopy. We found bassoon immunoreactivity in motor nerve terminals, concentrated in small puncta whose arrangement, number and alignment with junctional folds indicated that they were active zones (Fig. 5a). By contrast, antibodies to synaptic vesicle proteins stained nerve terminals more diffusely, as expected from ultrastructural studies. Moreover, the number of bassoon-immunoreactive puncta increased postnatally as the NMJ matured, in precise parallel with ultrastructural results (see below). Thus, anti-bassoon staining provides a new and convenient assay of active zones at the NMJ.

Active zones at synapses lacking laminin $\beta 2$ or Cav2.1

NMJs in mice lacking laminin $\beta 2$ (*Lamb2*^{-/-}) fail to mature properly, their synaptic clefts are invaded by Schwann cell processes, and their nerve terminals have a decreased number of active zones⁶⁻⁸. We asked whether mice lacking the Cav2.1 subunit shared any of these defects. Motor nerve terminals are rich in N-type (Cav2.2) channels at birth; these are replaced by Cav2.1 subunits during the

first few postnatal weeks. In $\text{Ca}_v2.1$ -deficient mutants, compensation by residual channels is incomplete, leading to progressive weakness and death in the third postnatal week^{25,27,28}. NMJs in $\text{Ca}_v2.1$ -deficient muscle were topologically normal, with varicose nerve terminals fully occupying an acetylcholine

receptor (AChR)-rich postsynaptic apparatus (Supplementary Fig. 6). Notably, laminin $\beta 2$ was normally concentrated in the synaptic cleft of $\text{Ca}_v2.1$ -deficient NMJs. With regards to ultrastructure, $\text{Ca}_v2.1$ -deficient nerve terminals bore normal concentrations of synaptic vesicles, the postsynaptic membrane was normally

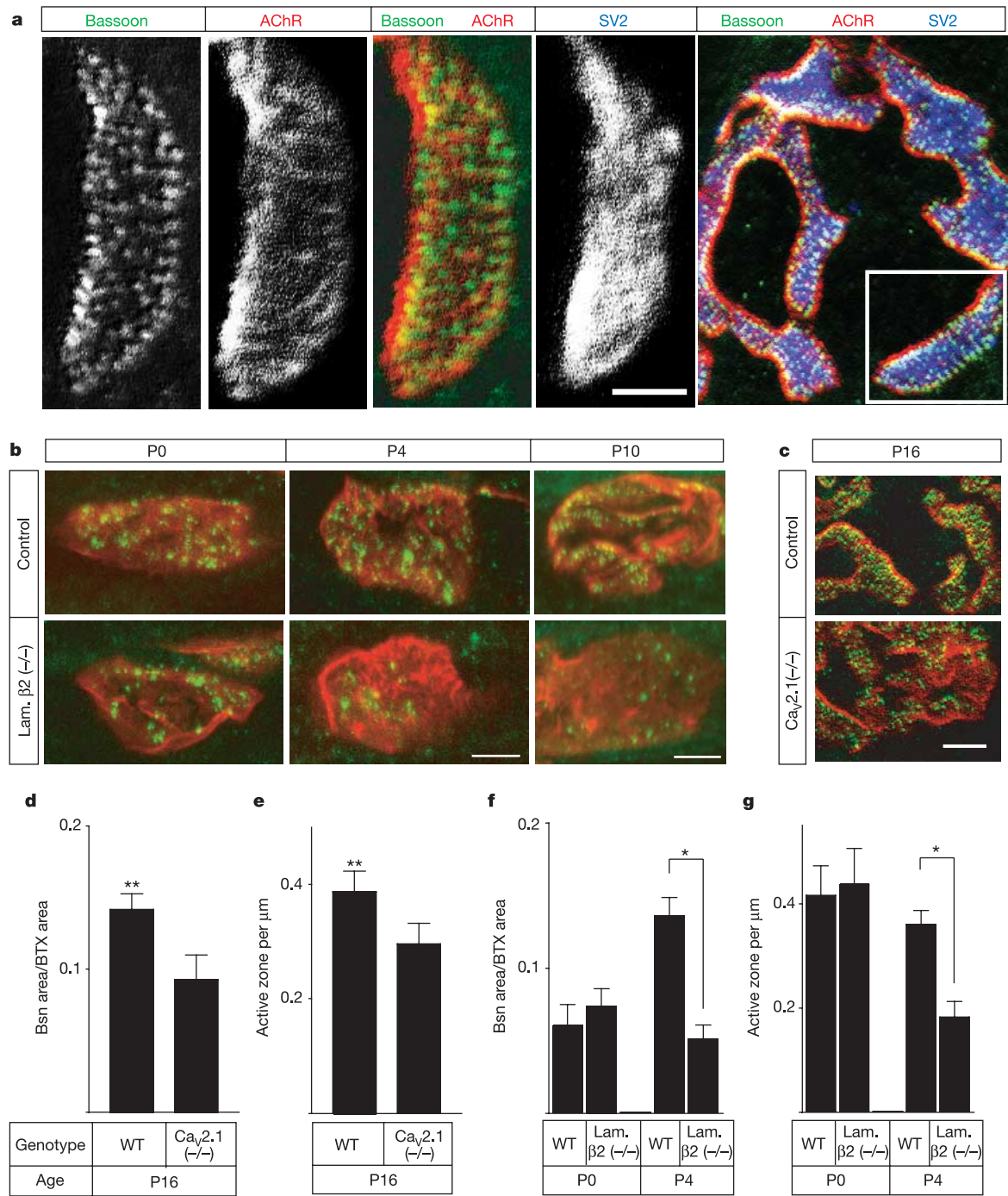


Figure 5 A new light microscopic assay for active zones used to monitor defects in laminin $\beta 2$ and $\text{Ca}_v2.1$ mutant NMJs. **a**, Adult NMJ stained in whole mount with anti-bassoon (green) to show active zones and Alexa-647- α -bungarotoxin (red) to label AChRs. The muscle was also stained with anti-SV2 (blue), showing more dispersed synaptic vesicles. The far right panel shows the whole NMJ from which high-magnification views (boxed region) were taken. No punctate staining was observed when control antibodies were substituted for anti-bassoon. **b–e**, Control, $\text{Lam}\beta 2^{-/-}$ and $\text{Ca}_v2.1$ -deficient NMJs of indicated postnatal ages were stained for bassoon (green) and AChRs (red). Examples are shown in **b, c**, and results are quantified in **d, f**. Graphs show bassoon (Bsn)-positive area

per unit AChR-rich (BTX) area. Calculation of puncta number instead of area gave similar results. **e, g**, Active zones at control, $\text{Lam}\beta 2^{-/-}$ and $\text{Ca}_v2.1$ -deficient-NMJs were also counted from electron micrographs (see Fig. 6 for example); graphs show number of active zones per μm of neuron–muscle apposition. Both light and electron microscopy show normal density of active zones in P0 $\text{Lam}\beta 2^{-/-}$ mice, and decreased numbers in older $\text{Lam}\beta 2^{-/-}$ and $\text{Ca}_v2.1$ -deficient mice. Each column represents 12–22 junctions in **d, f** and 51–199 profiles in **e, g**. Brackets indicate values that differ at $P < 0.0001$ (single asterisk) or $P < 0.04$ (double asterisk), Student's t -test. Scale bars: 2 μm (**a**), 5 μm (**b, c**). Graphs show mean $\pm$ s.e.m.

thickened and enfolded, and there was little if any invasion of the synaptic cleft by Schwann cell processes. However, $\text{Ca}_v2.1$ -deficient NMJs had a decreased number of active zones in their nerve terminals (Fig. 5c–e; see also Supplementary Fig. 6), a defect similar to that in $\text{Lamb}2^{-/-}$ mice. This result suggests that VGCCs are involved in formation or maintenance of active zones. The fact that $\text{Lamb}2^{-/-}$ NMJs are more severely defective than $\text{Ca}_v2.1$ -deficient NMJs may reflect the presence of residual $\text{Ca}_v2.2$ subunits in the latter^{25,28} and the fact that laminin $\beta 2$ has distinct and direct effects on Schwann cells⁷.

We also re-examined active zones in $\text{Lamb}2^{-/-}$ mice. Previous studies showed a reduced number of active zones by 1 week of age⁶, but presynaptic differentiation is already advanced in controls at this age, so the phenotype might reflect either a failure of nerve terminals to differentiate initially or to subsequently mature. To distinguish between these possibilities, we used electron microscopy and bassoon immunostaining in neonates. By both measures, the

density of active zones was normal in $\text{Lamb}2^{-/-}$ mice at birth, but significantly less than that in littermate controls by postnatal day 4 (P4; Fig. 5b, f, g). Importantly, an invasion of the synaptic cleft by Schwann cell processes, which physically separate pre- and post-synaptic membranes at later stages⁷, is minimal at P4 (data not shown), indicating that loss of active zones is not an indirect effect of synaptic detachment. These results suggest that laminin $\beta 2$ is dispensable for the initial formation of active zones, but is required for their maintenance or growth.

Laminin $\beta 2$ –VGCC interaction maintains active zones

The similar effects of $\text{Lamb}2^{-/-}$ and the $\text{Ca}_v 2.1$ mutation on active zone number are consistent with the notion that the laminin–VGCC interaction is essential for nerve terminal maturation. As a direct test, we attempted to block the interaction without affecting expression of either component. We injected mice with the $\text{Ca}_v2.1$ -derived loop peptide that inhibits binding of laminin $\beta 2$ to the VGCC (Fig. 3d). Mice were injected twice daily on P4 and P5 during a period of rapid synaptic growth. At P6, muscles were fixed and stained with anti-bassoon or prepared for electron microscopy. Both methods revealed that the number of active zones in $\text{Ca}_v2.1$ peptide-treated mice was $<50\%$ of that in untreated littermate controls (Fig. 6a–d). Vesicle number and polarization of the terminal were unaffected and peptide treatment had no obvious effect on the animals' health, voluntary movement, or synaptic size (Fig. 6e; see also Supplementary Fig. 7). As a control, we used the $\text{Ca}_v1.2$ -derived peptide that does not inhibit laminin $\beta 2$ –VGCC binding (Fig. 3d); this peptide had no effect on active zone number (Fig. 6c, d). These results indicate that laminin $\beta 2$ –VGCC interactions are critical for presynaptic development at the NMJ.

Discussion

Laminin and the VGCC are unexpected partners. Laminin has numerous effects on cellular differentiation, structure and function, but those analysed so far are mediated by signal-transducing receptors such as integrins, dystroglycan and immunoglobulin superfamily adhesion molecules⁹. The localization and activity of VGCCs are tightly controlled, but regulatory interactions documented so far involve the intracellular domains of the channel subunits^{16,17,29,30}. We have now shown that $\beta 2$ laminins exert at least some of their synaptic effects^{6–8,11,19} by binding directly to the VGCC pore-forming subunit. To our knowledge, the VGCC is the first channel shown to act as a laminin receptor, and laminins are the first extracellular proteins shown to be ligands for VGCCs.

Laminins are stably associated with the muscle fibre basal lamina, and the muscle can segregate $\beta 2$ laminins to portions of its surface that are rich in postsynaptic components, such as acetylcholine receptors³¹. By binding to VGCCs, immobilized laminin can anchor the channels to the membrane face that directly abuts the synaptic cleft. Intercellular domains of the channels, in turn, can interact with other presynaptic components, including vesicle and scaffolding proteins of active zones^{16,17,29,30}, thereby helping to organize neurotransmitter release sites. Further specificity may arise from the presence of multiple $\beta 2$ -containing laminin trimers at the synapse that differ in α -chains ($\alpha 2$, $\alpha 4$ or $\alpha 5$), each of which may have distinct receptors^{9,10}. For example, mice lacking laminin $\alpha 4$ retain active zones, but they are mislocalized³². Thus, synaptic laminins and VGCCs are parts of a multimolecular linkage that spans the synaptic cleft and extends into the nerve terminal. VGCCs thereby have key roles not only in mediating synaptic transmission but also in promoting the precise apposition of pre- to postsynaptic specializations.

Laminin $\beta 2$ –VGCC interactions may be required for formation of active zones or for their maintenance. We favour the latter possibility, because the number of active zones in peptide-treated mice at P6 was lower than that in control mice at P4, the time at which treatment was started. Taken together with the observation

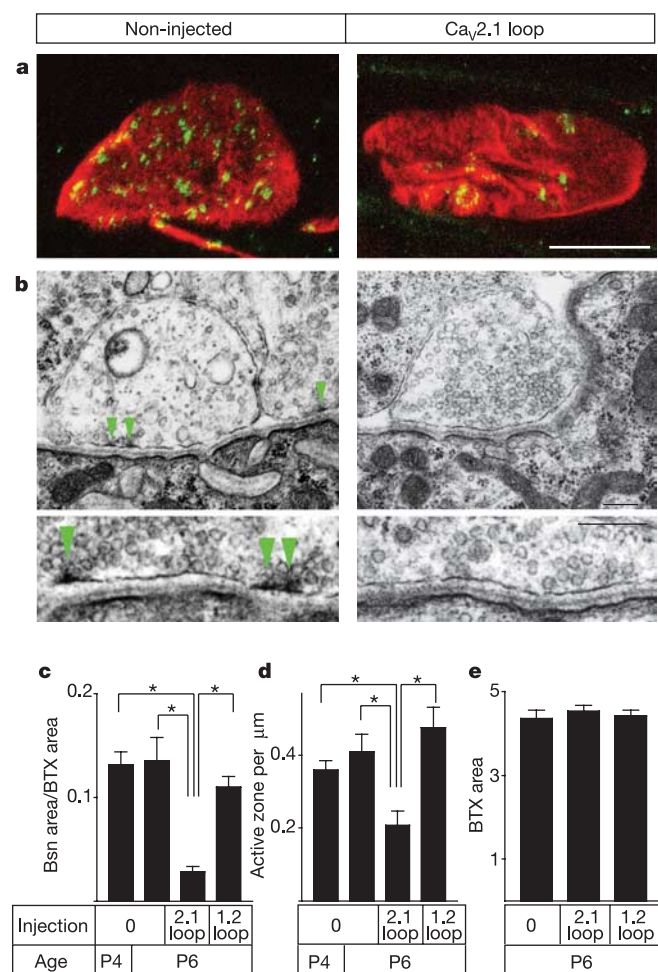


Figure 6 Blocking laminin $\beta 2$ –VGCC interactions *in vivo* disassembles active zones. **a, b**, Light (**a**) and electron microscopic (**b**) visualization of active zones in NMJs at P6. Left, control (non-injected); right, injected with $\text{Ca}_v2.1$ -derived peptide. Lower panels in **b** show higher-magnification views of a second example. Arrowheads mark active zones. Scale bars: $10\ \mu\text{m}$ (**a**), $250\ \text{nm}$ (**b**). **c, d**, Quantification of active zone density from micrographs such as those shown in **a** and **b**, respectively (see Fig. 5 legend for details). Values from P4 controls are re-drawn from Fig. 5. Injection of $\text{Ca}_v2.1$ -derived peptide but not $\text{Ca}_v1.2$ -derived peptide disassembled active zones. **e**, AChR-rich area, approximating synaptic size, was not affected by peptide treatment. Each y-axis unit is $50\ \mu\text{m}^2$. Each column represents 40–73 junctions from two (wild type, P6) and three (peptide-injected) animals in **c, e**, and 31–39 NMJ profiles in **d**. Asterisks indicate values that differ at $P < 0.0001$ (**c**) or $P < 0.007$ (**d**). Graphs show mean $\pm$ s.e.m.

that NMJs are not detectably perturbed at birth in *Lamb2*^{-/-} mice, these results suggest that laminin $\beta 2$ -VGCC interactions are required for the stability of active zones.

The effect of the Cav2.1-derived loop peptide on the molecular architecture of motor nerve terminals *in vivo* indicates that the integrity of active zones can be influenced in the short term by interactions with extracellular ligands such as laminin $\beta 2$. This result is noteworthy in two respects. First, motor nerve terminals can remodel rapidly, both during development and in response to changes in activity¹. The laminin $\beta 2$ -VGCC bond could be a control point for regulation of such remodelling. Second, disassembly of active zones is a characteristic feature of Lambert-Eaton myasthenic syndrome (LEMS), a disease of neuromuscular failure that results from an autoimmune attack on VGCCs of the NMJ^{33–36}. The accepted explanation for the pathogenesis of LEMS is that the antibodies lead to internalization of VGCCs, but our results suggest an additional possibility: that antibodies act in part by severing the laminin $\beta 2$ -VGCC bond. Consistent with this idea, sera of many LEMS patients have antibodies to the 11th extracellular loop of Cav2.1 (ref. 36), the region to which laminin $\beta 2$ binds.

Finally, Cav2.1- and Cav2.2-containing VGCCs are concentrated at central synapses as well as at NMJs, and are largely responsible for calcium-dependent neurotransmitter release from both types of nerve terminals^{16,17}. It is tempting to speculate that interactions of synaptic cleft components with extracellular sites on the VGCC are also important for active zone localization at central synapses³⁷. Little laminin $\beta 2$ is found at central synapses³⁸, but other proteins implicated in synaptogenesis and VGCC function, such as neuexins, are plausible candidates³⁹. The reagents and methods that we have reported here should be useful for seeking such ligands. □

Methods

Reagents

Sources of reagents and detailed methods for purification of laminin 9, preparation of protein-conjugated beads, generation of fusion protein and antibodies, and construction of chimaeric calcium channels are provided in the Supplementary Information.

Nearest-neighbour analysis of the VGCC-laminin complex

Synaptosomes from Torpedo electric organ were incubated in Sulfo-SBED (Pierce Chemical), pH 7.4 for 2 h in the dark at 4 °C, washed, irradiated with long wavelength light for 15 min and washed again. After solubilization with Triton X-100, extract was immunoprecipitated with anti-VGCC antibody. In some cases, the precipitated complex was dissociated by boiling in 1% SDS then re-precipitated with anti-HNK1, which is specific for laminin in this preparation¹³, before being subjected to western blot analysis. Biotinylated proteins were detected with streptavidin and biotinylated horseradish peroxidase (HRP) (Vectastain ABC kit, Vector Labs). Methods for synaptosome preparation, immunoprecipitation and western blotting are detailed in ref. 13.

Binding assays

To assay and map interactions between laminins and VGCCs, beads coated with laminins or laminin fragments were added to cultures of HEK293 cells that had been transfected (Lipofectamine 2000; Invitrogen) with expression vectors encoding VGCC subunits and cultured on poly-lysine-coated plastic. After incubation for 1 h at 37 °C, cells were washed, fixed with paraformaldehyde, and stained with anti-VGCC (Alomone Labs) and Alexa 568-conjugated second antibodies (Molecular Probes). We then counted the number of VGCC-positive cells with any adherent beads in ~20 microscope fields (40 $\times$); the number of VGCC-negative cells with any beads in the same fields was also determined.

To assay direct binding of laminin and VGCC fragments, alkaline phosphatase-laminin fusion proteins were incubated with His-tagged VGCC fragments. In a solid-phase assay, the VGCC fragments had been applied to nitrocellulose-coated wells of a 96-well dish and laminin fragments were added at 20–100 nM. In the solution assay, the laminin (500 nM) and VGCC fragments (8 μ M) were incubated for 1.5 h, after which Nickel-NTA resin (Novagen) was added; the precipitate was collected by centrifugation and washed. In both cases, binding of alkaline phosphatase fusion proteins was assayed as described in ref. 21.

Cell culture

Motor neurons were prepared from embryonic day 12.5 mouse spinal cord and cultured as described in ref. 22. After 2 days in culture, protein-coated beads were added; 3 days later, cells were fixed and stained. Where indicated, purified agatoxin-IVA (Sigma) and conotoxin-GVIA (Alomone) were added to the culture along with the beads.

Histological analysis

Laminin $\beta 2$ (*Lamb2*) and calcium channel Cav2.1 subunit mutant mice have been described previously^{6,27}. Methods for immunohistochemical analysis, electron microscopy

and morphometric analysis of electron micrographs are detailed in refs 10 and 32. Bassoon-positive area, puncta number and postsynaptic receptor area were determined in Metamorph (Universal Imaging Corporation) from confocal stacks by measuring the anti-bassoon-antibody-positive puncta overlying the bungarotoxin-labelled receptors.

Inhibitor injection

To assay laminin-VGCC interactions, mice were injected subcutaneously a total of four times at 12-h intervals on P4–5 with Cav1.2- or Cav2.1-derived fusion proteins described above (100 μ g g⁻¹ body weight per injection). Muscles were fixed at P6 and sectioned for light and electron microscopy as above. Both sternomastoid and gluteus muscles were analysed and gave similar results; data shown are from sternomastoid.

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Autonomous regulation of the anaphase-promoting complex couples mitosis to S-phase entry

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Oscillations in cyclin-dependent kinase (CDK) activity drive the somatic cell cycle. After entry into mitosis, CDKs activate the anaphase-promoting complex (APC), which then promotes cyclin degradation and mitotic exit. The re-accumulation of cyclin A causes the inactivation of APC and entry into S phase, but how cyclin A can accumulate in the presence of active APC has remained unclear. Here we show that, during G1, APC autonomously switches to a state permissive for cyclin A accumulation. Crucial to this transition is the APC^{Cdh1}-dependent autoubiquitination and proteasomal degradation of the ubiquitin-conjugating enzyme (E2) UbcH10. Because APC substrates inhibit the autoubiquitination of UbcH10, but not its E2 function, APC activity is maintained as long as G1 substrates are present. Thus, through UbcH10 degradation and cyclin A stabilization, APC autonomously downregulates its activity. This indicates that the core of the metazoan cell cycle could be described as a self-perpetuating but highly regulated oscillator composed of alternating CDK and APC activities.

The machinery of the somatic cell cycle integrates external signals with a basic oscillator, thereby coordinating events required for cell growth and division. Central to this machinery are cyclins, which activate specific kinases after association. During G1, D- and E-type cyclins convey exogenous signals to the basic cell cycle machinery by phosphorylating the retinoblastoma protein (pRb), leading to the activation of E2F transcription factors and entry into S phase. Surprisingly, neither D- or E-type cyclins nor the Cdk2 kinase is required for cell cycle progression in mammals^{1,2}. In contrast, cyclin A, expressed from late G1 until mitosis, is essential for both the G1/S and G2/M transitions^{3,4}.

Cyclin A binds and activates Cdk1 and Cdk2. Among its critical substrates at the G1/S transition is the anaphase-promoting complex/cyclosome (APC). APC is a multiprotein ubiquitin ligase that initiates sister chromatid separation at the metaphase/anaphase transition (reviewed in ref. 5). It also triggers exit from mitosis and is thought to remain active throughout G1. Because APC controls the degradation of proteins essential for DNA replication, its activity is downregulated before entry into S phase^{6,7}. APC inactivation also allows the accumulation of mitotic cyclins during S and G2, and thereby enables entry into the next mitosis. APC can be inhibited at the G1/S transition either by binding the Emi1 inhibitor or by phosphorylation of the APC activator Cdh1 by cyclin A/Cdk2 (refs 8–10). The expression of a non-phosphorylatable Cdh1 mutant, the ablation of cyclin A function in late G1 or the downregulation of Emi1 delay entry into S phase^{3,8,11}. Accordingly, heterologous expression of either cyclin A or Emi1 overcomes a G1 arrest mediated by constitutively active pRb^{8,9}. These results indicate that APC inactivation is required for S-phase entry and that it can be mediated by either cyclin A or Emi1.

Although cyclin A inactivates APC in G1, it is itself an APC substrate that is efficiently degraded in prometaphase^{12–16}. Degradation of cyclin A is required for proper chromosome congression, progression beyond metaphase, and establishment of G1 during *Drosophila* development. This raises a mechanistic paradox: if APC remains active throughout G1, how does cyclin A accumulate in late G1 and inhibit APC? Here we show that cyclin A can begin to accumulate only after the APC-specific ubiquitin-conjugating enzyme (E2) UbcH10 has been degraded. The cause

of the degradation of UbcH10 is its autoubiquitination, which is promoted by APC^{Cdh1}. We find that APC substrates inhibit the autoubiquitination of UbcH10 but not its E2 function; hence APC remains active until its substrates have been destroyed. APC therefore downregulates its activity through an autonomous mechanism centred on the degradation of a specific E2 enzyme. This mechanism couples the mitotic activation of APC to its inactivation before S-phase entry and suggests that the metazoan cell cycle is built around a self-perpetuating but highly regulated oscillator.

Cyclin A is stabilized after UbcH10 is degraded in G1

To study the activity of APC in G1, we prepared extracts from synchronized cells. The APC substrates securin, geminin and cyclin B1 were stable in extracts from nocodazole-arrested cells, which is consistent with the inhibition of APC by the spindle checkpoint (Supplementary Fig. S1a). In extracts from cells synchronized in G1, securin, geminin and cyclin B1 were degraded efficiently, and their degradation was inhibited by the competitive APC inhibitor N-cyclin B (NcycB). These extracts therefore recapitulate stage-specific APC activity. In contrast with the rapid and complete degradation of other APC substrates, cyclin A degradation was inefficient in these G1 extracts.

Because cyclin A is ubiquitinated by APC^{Cdh1} (ref. 11), it was assumed that cyclin A is unstable throughout G1. However, previous experiments did not distinguish between early and late G1 (refs 12, 17). To analyse whether cyclin A was stabilized at a specific stage during G1, we prepared extracts from cells that had been released from nocodazole arrest for various durations (Fig. 1a). Cyclin A was degraded in extracts from nocodazole-arrested cells in the presence of an active spindle checkpoint, although with lower efficiency (0 h; refs 12–14). When extracts were prepared from cells in late mitosis or early G1 (2 h), cyclin A rapidly turned over in an NcycB-sensitive and thus APC-dependent manner. Intriguingly, cyclin A degradation gradually diminished in later G1 extracts, whereas the APC^{Cdh1} substrates securin, geminin and Cdc20 were all still degraded. Thus, cyclin A degradation, which occurs rapidly in mitosis and in early G1, was blocked as cells progress through G1.

To identify limiting components for cyclin A degradation in mid/late G1, we supplemented G1 extracts with proteins known to

function with APC. Both E2 enzymes that interact with APC (UbcH5 α and UbcH10), but not their inactive mutants (UbcH5 α^{C85S} and UbcH10 $C114S$), promoted cyclin A degradation, even in late-G1 extracts (Fig. 1b). Cyclin A degradation was only minimally stimulated by UbcH10 in extracts of cells arrested in S phase, when APC is inactive (Fig. 1b). Thus, E2 enzymes are limiting for cyclin A degradation in mid/late G1.

In the reciprocal experiment we asked whether cyclin A degradation in early-G1 extracts is reduced by lowering E2 activity.

Immunodepletion of one of these E2 enzymes, UbcH10, inhibited cyclin A degradation almost as effectively as the APC inhibitor NcycB (Fig. 1c). The addition of UbcH10 to depleted extracts restored cyclin A degradation, just as it had supported cyclin A degradation in mid/late-G1 extracts. By contrast, UbcH10 depletion only weakly affected the degradation of other APC substrates, such as securin, indicating that the remaining UbcH5 is sufficient to catalyse securin but not cyclin A ubiquitination. Consistent with these observations was our finding that UbcH10 supported cyclin A

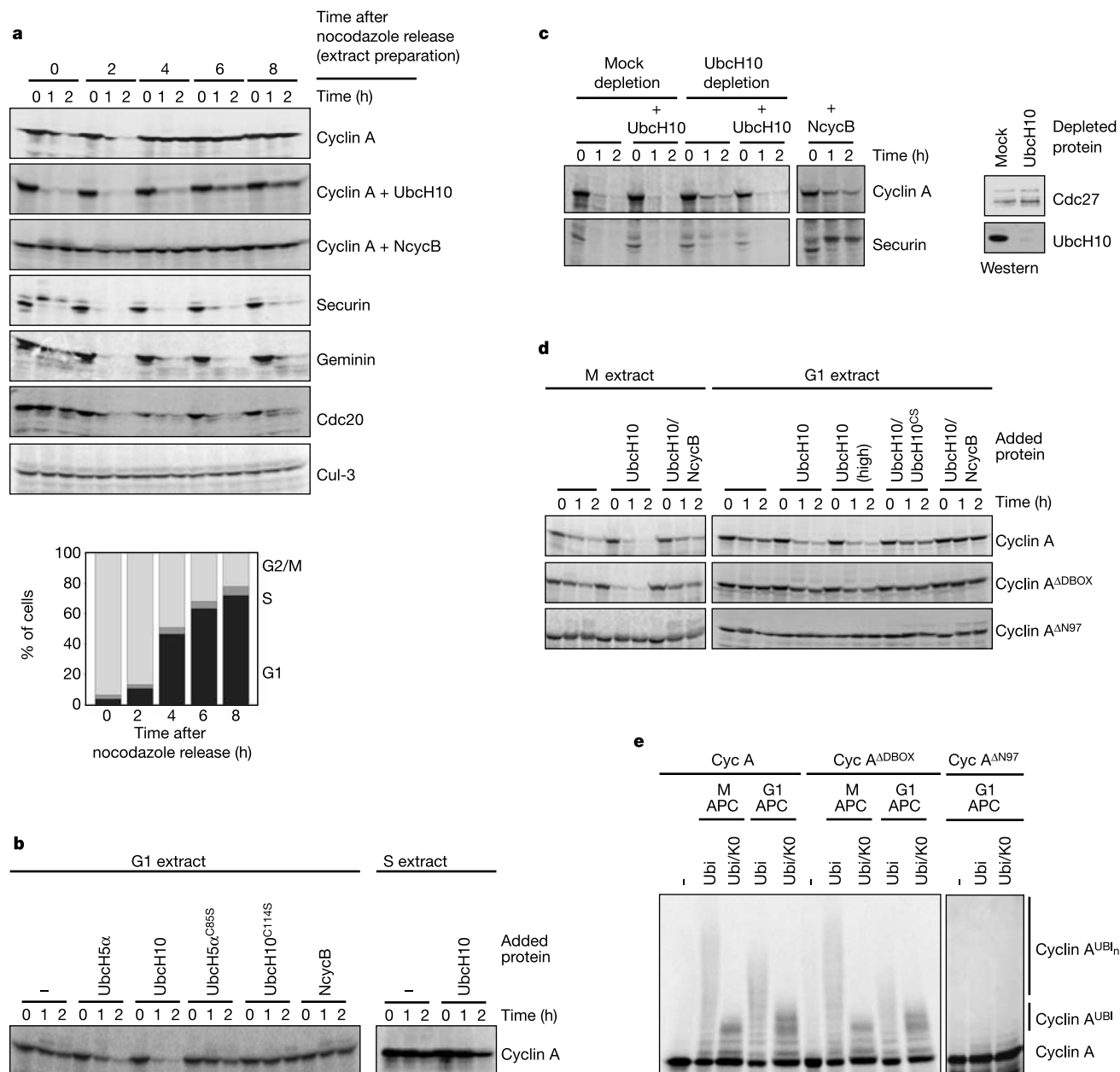


Figure 1 Cyclin A degradation is specifically impaired at low concentrations of UbcH10. **a**, The timing of cyclin A degradation in G1. Extracts were prepared at indicated times after release from arrest with nocodazole. The degradation of 35 S-labelled proteins was analysed over time by SDS-PAGE and autoradiography. The cell-cycle stage of the cells used for extract preparation as determined by fluorescence-activated cell sorting analysis is depicted below (light grey, G2/M; mid-grey, S; black, G1). **b**, Cyclin A degradation in G1 extracts (4 h after nocodazole release) is accelerated by the addition of UbcH10 or UbcH5 α , but not of inactive UbcH10 $C114S$ or UbcH5 α^{C85S} . Only minimal effects are observed in extracts of cells synchronized in S phase. **c**, Immunodepletion of UbcH10 in early G1 extracts (2 h after nocodazole release). Mock- or UbcH10-immunodepleted

extracts were analysed for degradation of 35 S-labelled cyclin A and securin. Immunoblots against the APC subunit Cdc27 and UbcH10 are shown on the right. **d**, Degradation of cyclin A, cyclin A Δ DBOX and cyclin A Δ N97 in mitosis and in G1. The degradation of 35 S-labelled proteins was analysed in extracts of cells arrested with nocodazole (M) or in G1 cells (G1). UbcH10 and NcycB were added as indicated. **e**, Ubiquitination of cyclin A, cyclin A Δ DBOX and cyclin A Δ N97 by APC Cdc20 (M APC) and APC Cdh1 (G1 APC). 35 S-labelled substrates were incubated with UbcH10, APC and either ubiquitin (Ubi) or a ubiquitin mutant lacking all lysines (Ubi/K0). The reaction products were resolved by SDS-PAGE and autoradiography.

ubiquitination by APC^{Cdh1} more efficiently than UbcH5 (Supplementary Fig. S1b). Taken together, these results indicate that cyclin A ubiquitination by APC^{Cdh1} is highly sensitive to the concentration of UbcH10.

The G1-specific ubiquitination of cyclin A by APC^{Cdh1} is different from its mitotic ubiquitination by APC^{Cdc20}. In mitotic extracts activated by UbcH10, a mutation of an amino-terminal D-box (cyclin A^{R47A/L50V}; referred to as cyclin A^{ΔDBOX}) had no effect on cyclin A stability (Fig. 1d; refs 12–14). However, in G1 the situation was different. Cyclin A^{ΔDBOX} (but not cyclin A) was stable in UbcH10-supplemented G1 extracts and not efficiently multi-ubiquitinated by APC^{Cdh1} (Fig. 1d, e). The multiubiquitination and degradation of cyclin A in G1 therefore require a conserved D-box and a threshold concentration of APC-specific E2 enzymes.

Because the degradation of cyclin A was very sensitive to the UbcH10 concentration in extracts, we analysed the concentrations of UbcH5 and UbcH10 throughout the cell cycle by western blotting and immunofluorescence (Fig. 2). In both experiments we found that UbcH10 concentration fluctuated, whereas UbcH5 concentration remained constant. UbcH10 accumulated with cyclin B1 and Plk1 as cells entered mitosis, and therefore its concentration was high at the time of cyclin A degradation (Fig. 2a). UbcH10 remained

stable during mitosis and in early G1 (Fig. 2b). Its concentration decreased only later in G1 with a significant delay compared with the APC^{Cdh1} substrates Cdc20 and Plk1, which were degraded in anaphase and telophase, respectively (Fig. 2b, c). The concentration of cyclin A began to increase shortly after UbcH10 was degraded. UbcH10 is therefore present in G1 extracts that degrade cyclin A, but its concentration declines before cyclin A accumulates late in G1. Together, these results indicate that the loss of UbcH10 in G1 stabilizes cyclin A, allowing it to accumulate in late G1.

APC-dependent regulation of UbcH10

Most importantly, UbcH10 is itself regulated by APC. APC^{Cdh1} purified from G1 cells, but not mitotic APC^{Cdc20}, efficiently catalysed UbcH10 multiubiquitination (Fig. 3a). Consistently, UbcH10 was rapidly degraded in G1 extracts by the proteasome but was stable in extracts of mitotic cells that were activated to degrade APC^{Cdc20} substrates by the addition of UbcH5 or UbcH10 (Fig. 3g). The multiubiquitination and degradation of UbcH10 in G1 were inhibited by both unlabelled UbcH10 and UbcH10^{C114S} in a concentration-dependent manner, which is consistent with a requirement for APC binding (Fig. 3a, b; Supplementary Fig. S2a). UbcH5 also inhibited APC-dependent UbcH10 ubiquitination.

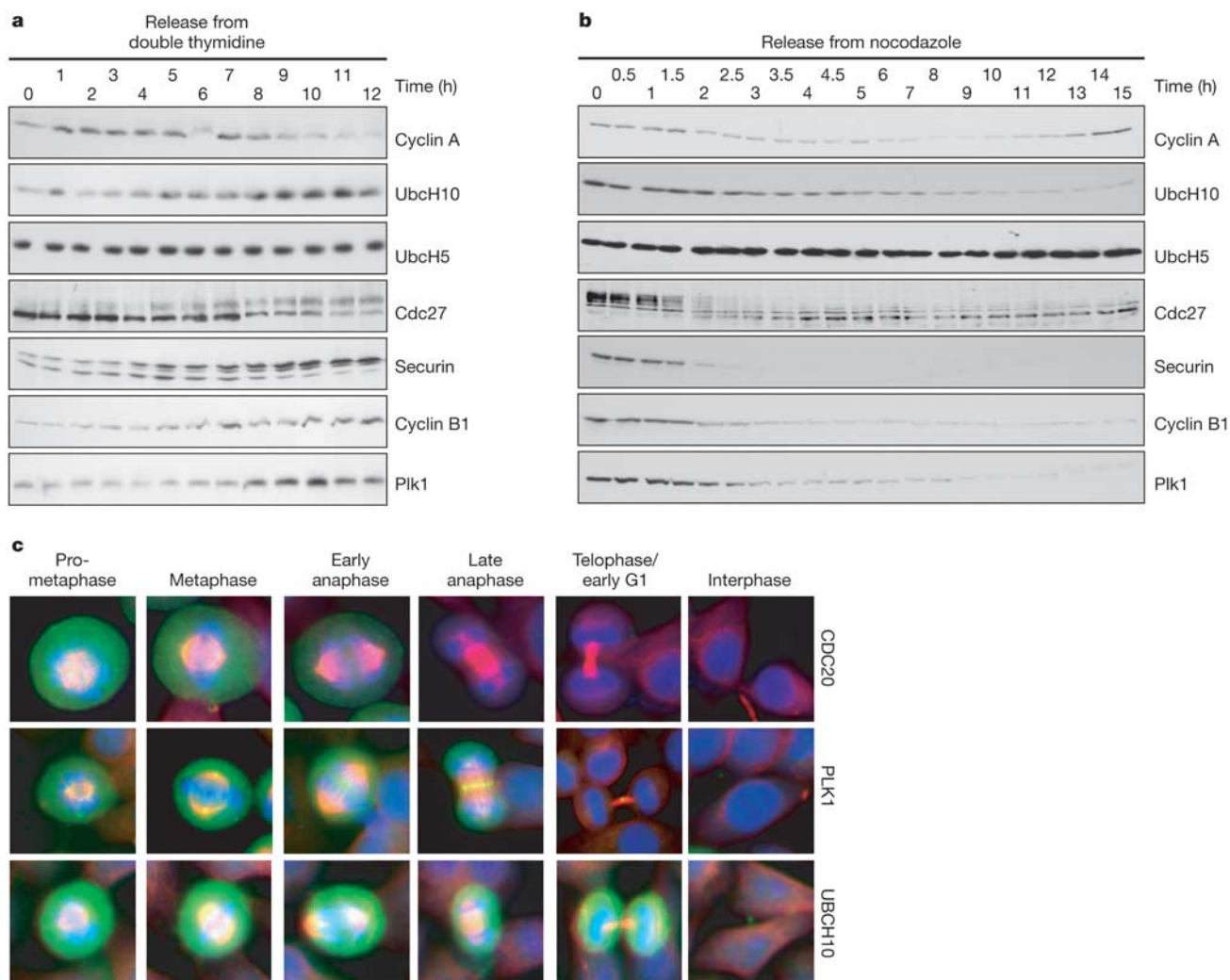


Figure 2 Cell-cycle regulation of E2 enzymes and cyclin A. **a**, Release of HeLa S3 cells from a double-thymidine arrest (early S phase) into nocodazole (mitosis). The expression of the depicted proteins was analysed by western blotting. **b**, Release of HeLa S3 cells from a nocodazole arrest, and analysis of protein expression by western blotting.

c, Analysis of UbcH10, Cdc20 and Plk1 expression (green) in asynchronous HeLa cells by immunofluorescence. Single representative cells of subsequent mitotic stages are depicted. β -Tubulin staining (red) indicates the formation of mitotic spindles. DNA is stained with 4,6-diamidino-2-phenylindole (blue).

This probably reflects the inability of APC to interact functionally with two different E2 enzymes at the same time, because both E2 enzymes require the APC11 subunit for ubiquitination¹⁸. In line with this interpretation, but in contrast with a previous report¹⁹, we found that UbcH10 multiubiquitination was strictly dependent on the activity of UbcH10 itself. Inactive UbcH10^{C114S} was not multiubiquitinated *in trans* by active UbcH10 or UbcH5 (Fig. 3a). Accordingly, UbcH10^{C114S} was not degraded in G1 extracts, but accumulated as a monoubiquitinated protein (Fig. 3g; data not shown). In summary, these results show that UbcH10 undergoes autoubiquitination *in cis* catalysed by APC^{Cdh1}, leading to its degradation in G1.

As mentioned above, there is a profound delay in the disappearance of UbcH10 relative to the onset of APC^{Cdh1} activity. At least two mechanisms contribute to this; intriguingly, both are intrinsic to APC. First, UbcH10 autoubiquitination *in cis* is significantly slower than geminin and cyclin A ubiquitination, both of which occur *in trans* (Fig. 3c). Second, other APC^{Cdh1} substrates interfere

strongly with UbcH10 autoubiquitination. Increasing amounts of NcycB and securin, both of which are APC^{Cdh1} substrates, inhibited the autoubiquitination of UbcH10 in a concentration-dependent manner, whereas non-substrate proteins had no effect (Fig. 3d; Supplementary Fig. S2b). Accordingly, in extracts both APC^{Cdh1} substrates stabilized UbcH10 (Fig. 3g). APC substrates therefore inhibit the slow autoubiquitination of UbcH10 and its degradation.

Surprisingly, these effects are not reciprocal. Whereas cyclin B and securin inhibited UbcH10 autoubiquitination, UbcH10 in turn did not block the ubiquitination of canonical APC substrates even when added at a 1,000-fold excess (Fig. 3e). Instead, increasing concentrations of UbcH10 further promoted the ubiquitination of APC^{Cdh1} substrates. By contrast, cyclin B and securin, both of which contain a well-characterized D-box, competed with APC^{Cdh1} substrates such as geminin in this assay (Fig. 3e). Consistently, high concentrations of UbcH10 did not impair APC-dependent degradation in G1 extracts, but stimulated it (data not shown). UbcH10 also did not interfere with substrate binding to APC^{Cdh1}; similarly,

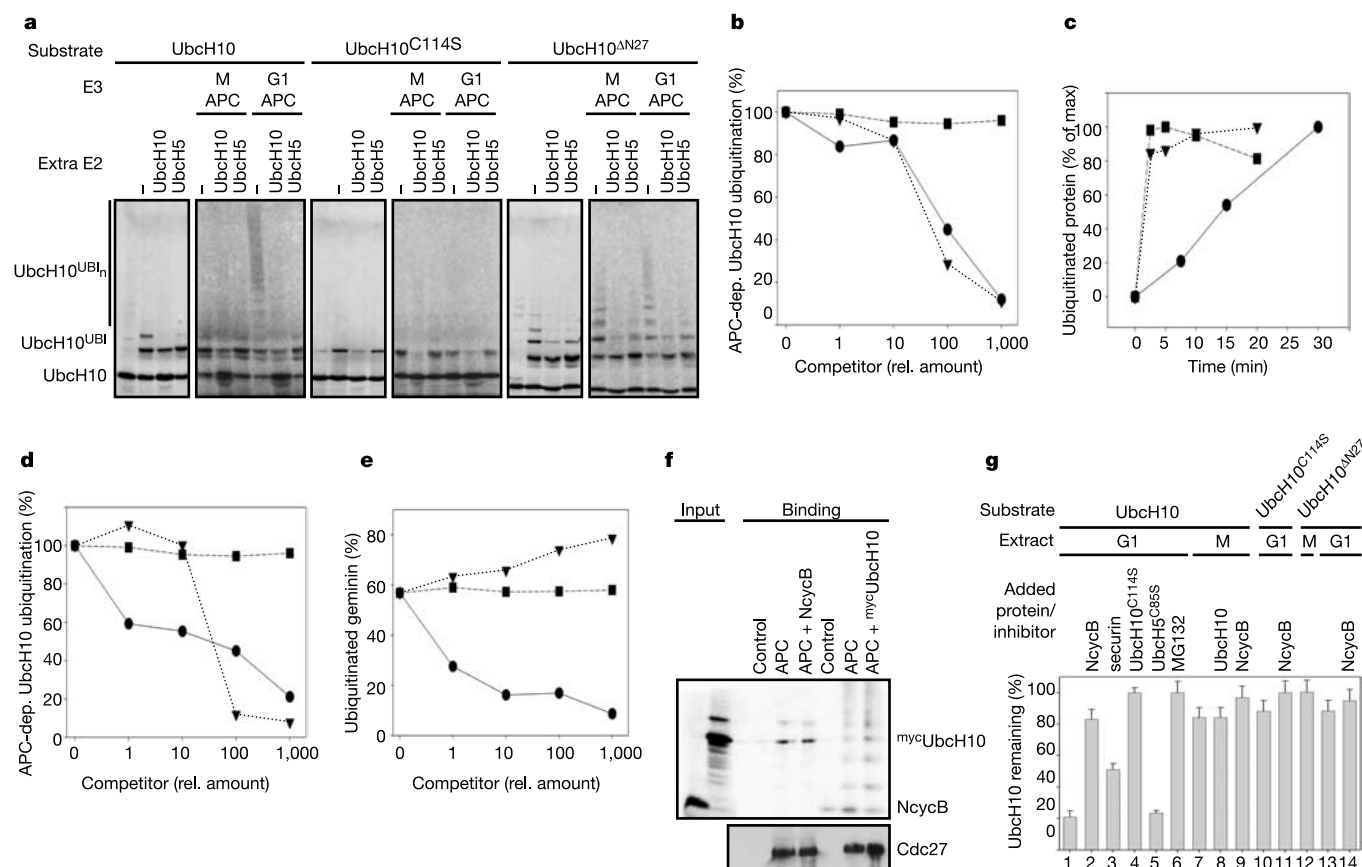


Figure 3 Degradation of UbcH10 in G1 is mediated by APC-dependent autoubiquitination. **a**, Analysis of UbcH10 autoubiquitination. ³⁵S-labelled UbcH10, UbcH10^{C114S} and UbcH10^{ΔN27} were incubated with buffer alone, with E1 (–) or with APC from nocodazole-arrested (M APC) or G1 (G1 APC) cells. Unlabelled E2 was added as indicated, and reaction products were analysed by autoradiography. Monoubiquitination of all UbcH10 mutants (UbcH10^{UBI}) is observed after the addition of E1. Multiubiquitination (UbcH10^{UBI}) depends on UbcH10 activity and on G1 APC. **b**, Regulation of UbcH10 autoubiquitination by APC binding. Autoubiquitination of ³⁵S-labelled UbcH10 was analysed as described, and increasing concentrations of unlabelled UbcH10 (circles), UbcH10^{C114S} (triangles) or Mad2 (squares) were added. **c**, Kinetics of UbcH10 autoubiquitination. UbcH10 autoubiquitination (circles) was performed in the presence of G1 APC, and aliquots were removed at the indicated times. Similarly, ubiquitination of geminin (triangles) and cyclin A (squares) by APC^{Cdh1} in the presence of UbcH10 was analysed. **d**, APC substrates inhibit UbcH10 autoubiquitination. ³⁵S-labelled UbcH10 was incubated with G1 APC in the presence of increasing amounts of unlabelled APC substrates N-cyclin B1 (circles) and

securin (triangles), and of the control protein Mad2 (squares). **e**, UbcH10 does not inhibit degradation of APC substrates in G1. ³⁵S-labelled geminin was incubated with APC^{Cdh1}, UbcH5 and increasing concentrations of unlabelled NcycB (circles), UbcH10 (triangles) or Mad2 (squares). The percentage of ubiquitinated geminin is indicated. **f**, UbcH10 and substrates bind APC simultaneously. The amount of ³⁵S-labelled UbcH10 and NcycB bound to IgG beads (control) or APC^{Cdh1} in the presence or absence of a 1,000-fold excess of the respective other unlabelled protein was determined by autoradiography. APC was detected by western blotting against Cdc27. **g**, Degradation of UbcH10, but not of UbcH10^{C114S} and UbcH10^{ΔN27}, occurs in G1. Untagged ³⁵S-labelled proteins were generated from ProA/TEV-UbcH10, ProA/TEV-UbcH10^{C114S} and ProA/TEV-UbcH10^{ΔN27} by cleavage with TEV protease and incubated in extracts prepared from nocodazole-arrested cells (M) or from cells released into G1 for 4 h. The protein concentrations after 120 min were quantified and compared with the initial concentrations. Unlabelled proteins or the proteasome inhibitor MG132 were added as indicated. Results are means ± s.e.m. for three independent experiments.

substrates did not affect the binding of UbcH10 to APC^{Cdh1} (Fig. 3f). UbcH10 therefore has little affinity for the substrate-binding site on APC. This enables substrates to selectively inhibit the autoubiquitination of UbcH10, but not its E2 function, thereby preventing premature APC inactivation.

The autoubiquitination of UbcH10 is regulated by sequence motifs within the N-terminal extension of UbcH10, which is unique among E2 enzymes. Its deletion (UbcH10^{ΔN27}) impaired the formation of ubiquitin chains by APC^{Cdh1} but simultaneously allowed some ubiquitination of UbcH10 by mitotic APC^{Cdc20} (Fig. 3a). However, these ubiquitin chains did not promote the proteasomal degradation of UbcH10^{ΔN27} in extracts (Fig. 3g). A similar misregulation of both ubiquitination and degradation was observed with N-terminally tagged UbcH10 (data not shown). The integrity of its N terminus is therefore required for the APC-dependent autoubiquitination of UbcH10 in G1 and the prevention of its multiubiquitination in mitosis. The N-terminal extension of UbcH10 is conserved in all metazoan homologues but not in yeast. Yeasts also lack a clear cyclin A homologue, raising the

interesting possibility that cyclin A and UbcH10 might have coevolved.

APC therefore promotes the autoubiquitination of UbcH10 *in cis*. This is in contrast with a report that implied that UbcH10 is a D-box-dependent APC substrate¹⁹. However, these experiments were performed mainly with N-terminally tagged UbcH10, which as shown here interferes with the regulation of UbcH10 autoubiquitination. In addition, mutation of the proposed D-box produces an inactive protein incapable of catalysing its own ubiquitination¹⁹.

Regulation of cyclin A accumulation by UbcH10 *in vivo*

To study the effects of UbcH10 on cyclin A accumulation *in vivo* we expressed mycUbcH10, which is stabilized because of its N-terminal tag. We could not synchronize cells by mitotic arrest, because UbcH10 overexpression itself abrogated the checkpoint (data not shown). When cells expressing mycUbcH10 were instead synchronized at the G1/S transition, the concentration of cyclin A was decreased (Fig. 4a). This could not be due solely to effects on cyclin A transcription, because cyclin A expressed from a constitutive

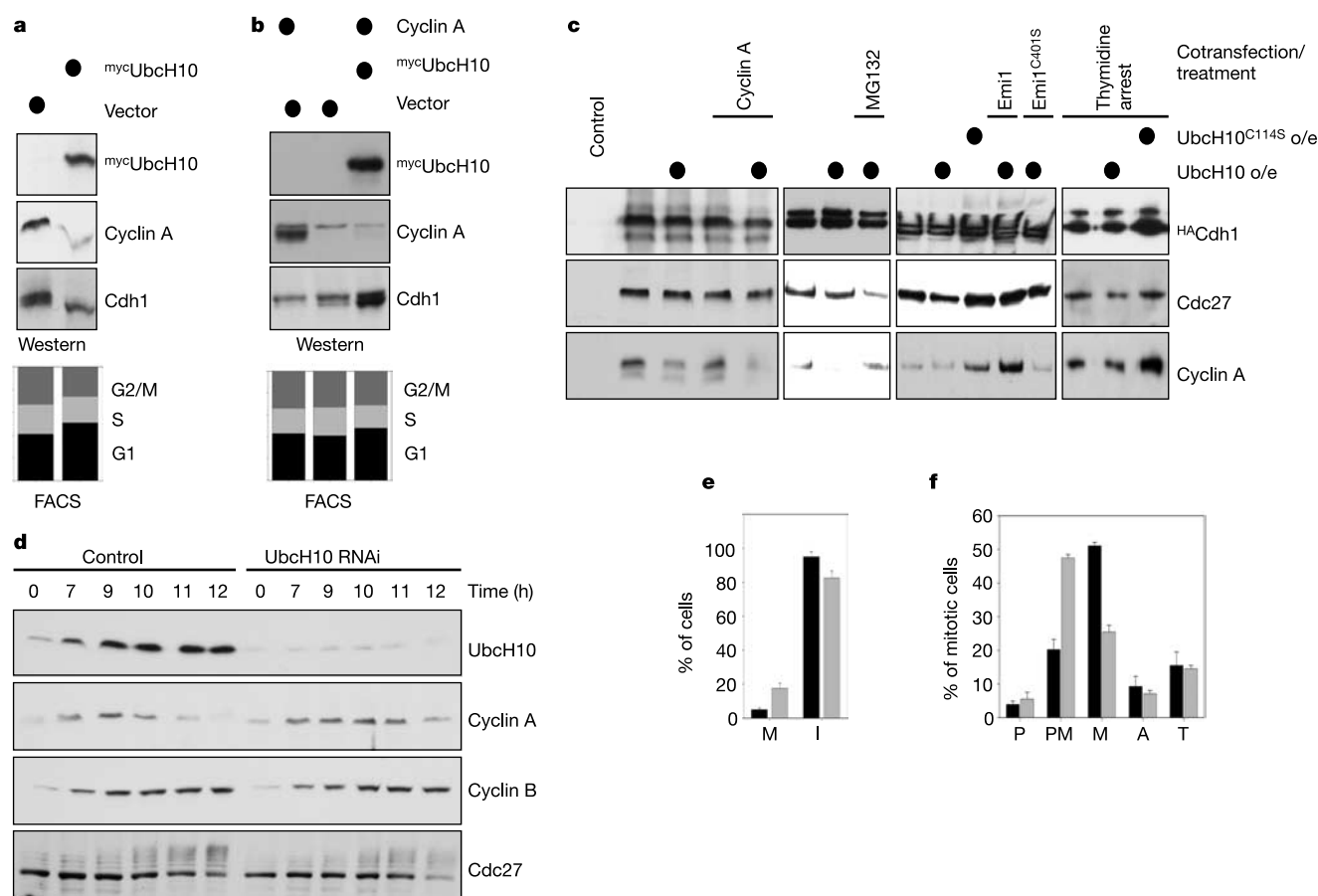


Figure 4 Misregulation of UbcH10 degradation interferes with cyclin A accumulation. **a**, Overexpression of stable mycUbcH10 impairs cyclin A accumulation at the G1/S transition. Cells were transfected with a plasmid expressing mycUbcH10 and subjected to a double-thymidine arrest. Cyclin A and mycUbcH10 were detected by western blotting. The cell-cycle stage of transfected cells was determined by sorting with green fluorescent protein (GFP) and fluorescence-activated cell sorting (FACS). **b**, Coexpression of cyclin A and mycUbcH10 interferes with cyclin A accumulation in early S phase. Cells were transfected with the indicated constructs, synchronized in early S phase by a double-thymidine arrest, and analysed by western blotting. The cell cycle stage of transfected cells was determined by GFP sorting and FACS. **c**, Overexpression (o/e) of UbcH10 inhibits the binding of cyclin A to Cdh1. Cells were transfected with HA-Cdh1, mycUbcH10, mycUbcH10^{C114S}, mycEmi1 and mycEmi1^{C401S} as indicated. Cdh1-containing complexes were purified by anti-HA immunoprecipitation from asynchronously grown cells or from

cells arrested in S phase by thymidine treatment. The proteasome inhibitor MG132 was added to cultures 2 h before harvesting when indicated. Cyclin A and Cdc27 were detected in HA-Cdh1 precipitates by western blotting. **d**, Depletion of UbcH10 by RNAi delays cyclin A degradation in prometaphase. Cells were treated with control siRNA or siRNA against UbcH10, arrested in early S phase, and released into mitosis in the presence of nocodazole. Samples were taken at the indicated times and analysed by western blotting. **e**, HeLa cells were treated with either control siRNA (dark bars) or siRNA against UbcH10 (light bars), and mitotic (M) and interphase (I) cells were counted. **f**, Mitotic cells of HeLa cells either treated with control siRNA (dark bars) or siRNA against UbcH10 (light bars) were categorized into prophase (P), prometaphase (PM), metaphase (M), anaphase (A) and telophase (T) by cell morphology and by immunostaining against cyclin A, cyclin B, Cdc20 and Plk1. Results in **e** and **f** are means $\pm$ s.e.m. for three independent experiments.

promoter was equally downregulated by $^{myc}UbcH10$ (Fig. 4b). However, these effects are transient. When the cyclin A-expressing cells were released from the arrest at the G1/S transition into the next cell cycle, cyclin A reaccumulation was delayed, but not blocked, by $^{myc}UbcH10$ (Supplementary Fig. S3a). Consistently, the expression of $^{myc}UbcH10$ caused a decrease in the fraction of S-phase cells in an asynchronous population, but it did not permanently arrest cells in G1 (Supplementary Fig. S3b). These experiments show that UbcH10 promotes cyclin A degradation *in vivo*, but also imply that backup mechanisms exist that drive cells into S phase in the event of UbcH10 misregulation.

We repeated these experiments and directly measured the binding of cyclin A to tagged Cdh1. In asynchronous cells, the expression of $^{myc}UbcH10$, but not that of inactive $^{myc}UbcH10^{C114S}$, decreased the amount of cyclin A co-precipitating with Cdh1, reflecting the decreased cyclin A concentration (Fig. 4c). By contrast, cyclin A concentration remained high in Cdh1 precipitates, when the proteasome was inactivated by MG132, or in S-phase cells, in which APC is inactive. Similarly, coexpression of Emi1 counteracted any effects of UbcH10 overexpression. UbcH10 therefore induced the degradation of cyclin A *in vivo* in an APC^{Cdh1}- and proteasome-dependent manner. Importantly, after UbcH10 has been degraded, cyclin A can bind Cdh1 stably.

To study the consequences of UbcH10 depletion, we decreased UbcH10 concentration by using short interfering RNA (siRNA). Significant effects occur in prometaphase, in which cyclin A degradation was significantly delayed (Fig. 4d). Accordingly, UbcH10 depletion led to a threefold increase in the mitotic index and in the percentage of cyclin A-positive prometaphase figures, which is indicative of a delay in mitotic progression at prometaphase (Fig. 4e, f). This caused mitotic aberrations, as judged by a fourfold increase in multinuclear cells and the presence of cells with multipolar spindles and misaligned DNA in the siRNA-treated samples (Supplementary Fig. S4a). Similar phenotypes were reported after overexpression of cyclin A or Emi1, suggesting that

the depletion of UbcH10 could have caused these mitotic aberrations by inhibiting cyclin A degradation^{12,13,16}.

However, unlike the situation in G1, the decrease in UbcH10 concentration in mitosis did not permit the selective degradation of other APC substrates, while maintaining cyclin A stability. In UbcH10-depleted mitotic cells, securin, cyclin B, Cdc20 or Plk1 were stable until cyclin A had finally been degraded (Supplementary Fig. S4b). This might be due to differences between cyclin A degradation during mitosis and G1, or to a requirement for cyclin A degradation to permit full APC activation towards other substrates.

Taken together, these experiments show conclusively that UbcH10 is rate-limiting for cyclin A degradation both *in vitro* and *in vivo*. The increase in UbcH10 concentration in mitosis is required for efficient cyclin A degradation in prometaphase. The degradation of UbcH10 in G1 accordingly stabilizes cyclin A and eventually allows the complete inactivation of APC.

Emi1, UbcH10, and S-phase entry

Cells commit to a complete round of cell division during G1 in response to exogenous growth factors. The autonomous inactivation of APC through UbcH10 degradation should therefore be coupled to external signals. A potential mediator of this coupling is Emi1, which is transcribed at the G1/S transition in an E2F-dependent manner and can inactivate APC^{Cdh1} independently of Cdh1 phosphorylation⁸. Indeed, Emi1 also inactivates APC in the presence of high concentrations of UbcH10. As shown in Fig. 5a, when the Zn-binding domain of Emi1, but not that of inactive Emi1^{C401S}, was added to G1 extracts supplemented with UbcH10, cyclin A degradation was blocked. Similarly, Emi1, but not Emi1^{C401S}, Mad2 or Mad2L2, inhibited the APC^{Cdh1}-dependent ubiquitination of cyclin A and geminin in the presence of UbcH10 (Fig. 5b). Both Emi1 and Emi1^{C401S} also inhibited the autoubiquitination of UbcH10. Finally, Emi1 counteracts UbcH10 *in vivo*, as shown by the increased amount of Cdh1-bound cyclin A in cells

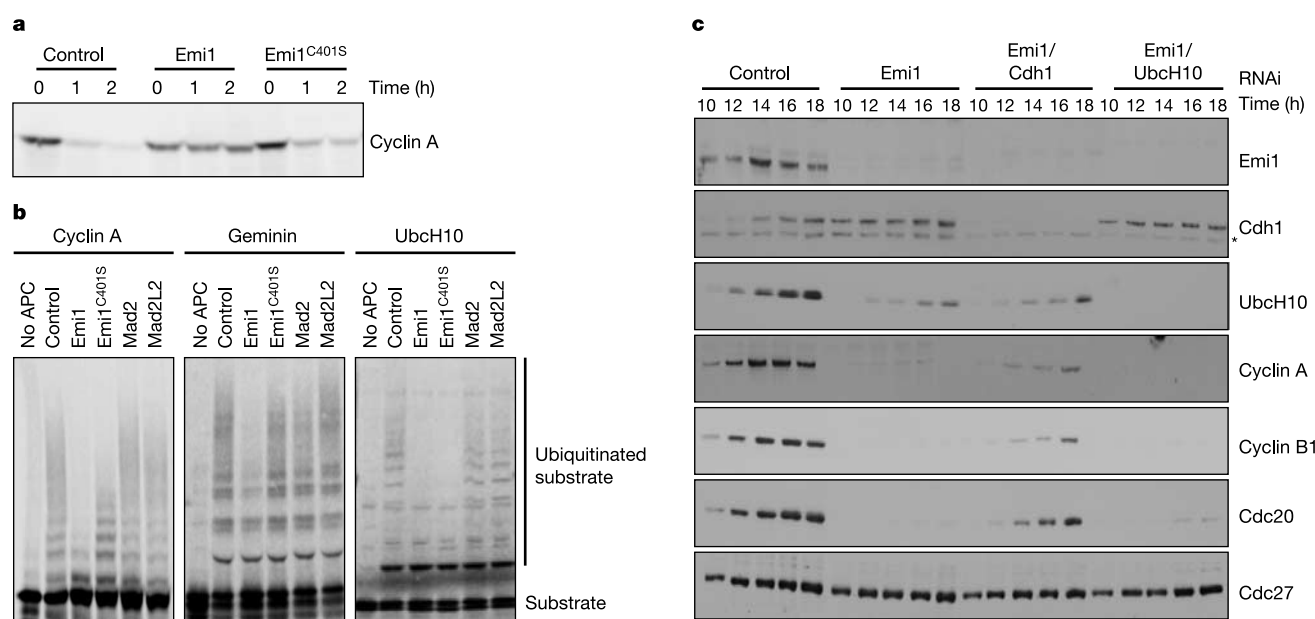


Figure 5 Control of autonomous APC inactivation by Emi1. **a**, Emi1 inhibits cyclin A degradation in the presence of high concentrations of UbcH10. G1 extracts were supplemented with UbcH10 and with a C-terminal fragment of Emi1 or of inactive Emi1^{C401S}. The degradation of ³⁵S-labelled cyclin A was analysed as before. **b**, Emi1 inhibits both substrate ubiquitination and UbcH10 autoubiquitination. APC^{Cdh1} from G1 cells was analysed for its capacity to catalyse the ubiquitination of either cyclin A or geminin, or the autoubiquitination of UbcH10, respectively. C-terminal fragments of Emi1

and Emi1^{C401S}, or Mad2 and Mad2L2, were added as indicated. **c**, Emi1 is required for entry into S phase in the absence of Cdh1. Cells treated with siRNAs against Emi1, Cdh1 and UbcH10, as indicated, were released from a nocodazole arrest. Samples were taken after the indicated durations, and entry into S phase was measured by western blotting against the indicated proteins. The asterisks mark nonspecific bands of the respective antibodies.

expressing Emi1 and UbcH10 (Fig. 4c). Emi1 is therefore a general APC inhibitor that could inactivate APC in the event of UbcH10 misregulation, or during S and G2, when UbcH10 concentrations recover.

However, the role of Emi1 at the G1/S transition might be more complex. When Emi1 was depleted by siRNA, cells were arrested at the G1/S transition (Fig. 5c; ref. 8). This might be caused partly by an unexpected increase in Cdh1 concentration, because elevated Cdh1 concentrations would overwhelm the low cyclin A concentrations present before entry into S phase^{11,20}. Most significantly, however, when both Cdh1 and Emi1 are depleted by siRNA, cells were still arrested in G1 (Fig. 5c). Thus, even when APC has been inactivated by UbcH10 degradation and Cdh1 depletion, Emi1 is required for S-phase entry. This indicates that there might be an additional, hitherto unknown, S-phase-promoting function of Emi1, which is independent of APC^{Cdh1} and connects the final steps of S-phase entry to complete E2F activation. APC therefore first downregulates its activity towards cyclin A by UbcH10 auto-ubiquitination, allowing cyclin A to accumulate. This is followed by the E2F-dependent transcription of cyclin A and Emi1, which ensures complete inactivation of APC^{Cdh1} and promotes S phase by additional means.

Discussion

Cyclin A is the only essential cyclin for S-phase entry in unperturbed cell cycles^{1–3}. It inactivates APC by phosphorylating Cdh1, and it regulates DNA replication. However, cyclin A is also a substrate of APC, and the cyclin-binding motif of Cdh1 is pivotal for both Cdh1 phosphorylation and cyclin A ubiquitination^{10,11}. An explanation of why, early in G1, cyclin A is ubiquitinated by APC^{Cdh1} but stably binds and inactivates APC^{Cdh1} late in G1 has long remained elusive. Here we show that the decision between cyclin A degradation and APC inactivation is determined by the availability of UbcH10. UbcH10 is rate-limiting for the G1-specific ubiquitination of cyclin A. The degradation of UbcH10 consequently stabilizes cyclin A, which then can bind to Cdh1 without being degraded. APC^{Cdh1} itself controls this transition by promoting the autoubiquitination of UbcH10 *in cis*. Other substrates of APC act as timers and inhibit the autoubiquitination of UbcH10 but not its E2 activity. This ensures that mitotic proteins are degraded before APC is inactivated. Once activated in mitosis, APC therefore autonomously downregulates its activity through the ubiquitination and degradation of UbcH10.

These findings have important implications for our understanding of the eukaryotic cell cycle. The autonomous inactivation of APC strongly suggests the existence of a self-perpetuating oscillator at the core of eukaryotic cell cycles. This oscillator would be composed only of the APC ubiquitin ligase and two cyclin/CDKs. In this simplified scheme, cyclin A/Cdk1 and cyclin B/Cdk1 promote entry into mitosis and activate APC²¹; APC in turn inactivates mitotic cyclins and triggers exit from mitosis; degradation of its remaining substrates is followed by the autoubiquitination and degradation of UbcH10; subsequent accumulation of cyclin A completely inactivates APC, promotes S-phase entry and allows the reaccumulation of mitotic cyclins and UbcH10.

In multicellular organisms, this self-perpetuating oscillator is tightly coupled to external controls. In addition to the degradation of UbcH10 and the stabilization of cyclin A, transcriptional steps are required for entry into S phase. These include an increased transcription of not only cyclin A but also Emi1 (refs 8, 9). Emi1 combines an uncharacterized APC^{Cdh1}-independent activity, essential for S-phase entry, with its role as an APC inhibitor. Why the cell requires a further means of inhibiting APC after UbcH10 degradation and Cdh1 phosphorylation seems surprising. One possibility, in addition to the need for triple assurance, is that inactivation of APC by Emi1 is needed in S and G2, when UbcH10 and activators of APC reaccumulate. Furthermore, Emi1 could

assist in Cdh1 inactivation in case cyclinA/Cdk2 activity is restrained by CDK inhibitors or UbcH10 is misregulated. The cell cycle therefore seems to be composed of an endogenous oscillator driven largely by post-translational mechanisms, namely phosphorylation and degradation, and of multiple transcriptional and translational controls that couple this oscillator to exogenous signals. It will be interesting to examine variations on this theme in different organisms. □

Methods

Plasmids and antibodies

UbcH10 and UbcH10^{C114S} were cloned into pCS2^{ProA/TEV} for *in vitro* transcription/translation, or into pET28 for purification. Cdh1 was cloned into haemagglutinin-tagged pCS2 to generate HA-Cdh1. Securin, geminin, Cdc20 and cyclin A were all in pCS2. myc-Emi1 and myc-Emi1^{C401S} were in pCS2. Carboxy-terminal fragments of Emi1 and Emi1^{C401S} were cloned into pET28. Anti-UbcH10 and anti-UbcH5 antibodies were purchased from Boston Biochem. Anti-Cdc27, anti-HA, anti-cyclin A, anti-cyclin B1 and anti-Cdc20 antibodies were purchased from Santa Cruz. Anti-securin antibody was purchased from MBL, and anti-Plk1 antibody from Upstate. Anti-Emi1 antibody was a gift from P. Jackson. Alexa 488-labelled and Alexa 546-labelled secondary antibodies were purchased from Molecular Probes, and Cy3-labelled anti-β-tubulin antibody from Sigma.

Tissue culture and cell synchronization

HeLa, HeLa S3 and 293T cells were synchronized in prometaphase by treatment with nocodazole, in G1 by a release from nocodazole arrest, and in early S by double-thymidine arrest. Cells were incubated in thymidine-containing (2 mM) medium for 24 h. Cells were released into fresh medium for 6 h, followed by a second thymidine arrest for 18 h or a nocodazole arrest (0.1 μg ml⁻¹) for 12 h. For G1 cells, nocodazole-arrested cells were released into fresh medium for 4 h unless noted otherwise. Cells were harvested, washed with PBS, and either processed for extraction or lysed in SDS buffer for analysis by SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) and western blotting.

Protein purification

His-UbcH10, His-UbcH10^{C114S}, His-tagged Zn-binding domains of Emi1 and Emi1^{C401S}, and His-Mad2 were purified from BL21(DE3) pRIL cells (Stratagene) by Ni²⁺-nitrilotriacetate agarose chromatography in accordance with the manufacturer's protocol (Qiagen). MBP-Mad2L2 was purified after expression in BL21(DE3) pRIL cells on amylose resin according to the manufacturer's protocol (NEB).

Degradation assays

In vitro degradation assays were performed with extracts of synchronized HeLa S3 cells. Synchronized cells were harvested, washed with PBS, lysed in SB buffer (25 mM HEPES pH 7.5, 1.5 mM MgCl₂, 5 mM KCl, 1 mM dithiothreitol, 1 × complete protease inhibitors (Roche), 15 mM creatine phosphate, 2 mM ATP) and homogenized by freeze-thawing and passage through a needle. Extracts were cleared by subsequent centrifugations (5 min at 5,000 r.p.m.; 60 min at 14,000 r.p.m.). Extract (20 μl) was supplemented with degradation cocktail (1.5 mg ml⁻¹ ubiquitin (Boston Biochem), 7.5 mM creatine phosphate, 1 mM ATP, 1 mM MgCl₂, 0.1 mg ml⁻¹ cycloheximide) and ³⁵S-labelled substrate at 23 °C. Aliquots were removed at 0, 1 and 2 h, and resolved by SDS-PAGE (5–15%) and autoradiography. To analyse the degradation of UbcH10, it was synthesized as ProA/TEV-UbcH10, and 1 U of tobacco etch virus (TEV) protease was included in the degradation assay.

Ubiquitination assays

In vitro ubiquitination assays were performed with APC purified from extracts of synchronized HeLa S3 cells. For 10 ubiquitination reactions, 1 ml of extract was incubated with 10 μg of anti-Cdc27 antibodies for 4 h at 4 °C, and with ProG-agarose (Roche) for 2 h. Precipitates were washed three times with SB buffer containing 0.1% Triton X-100 and twice with SB buffer. E1 (Boston Biochem), E2 (as indicated), 20 mM ATP, 1.5 mg ml⁻¹ ubiquitin and 10 mM dithiothreitol in UBAB buffer (25 mM Tris/HCl pH 7.5, 50 mM NaCl, 10 mM MgCl₂) were added. Reactions were started by the addition of ³⁵S-labelled substrate, incubated for 60 min at 30 °C and resolved by SDS-PAGE and autoradiography.

Immunodepletion of UbcH10

Synchronized HeLa S3 cells (500 ml) were used for extraction as described above. The extract was incubated for 2 h with 10 μg of anti-UbcH10 antibody coupled to ProG-agarose. Beads were removed by centrifugation, and supernatants were used in degradation assays as described above.

Immunofluorescence

HeLa cells were grown on coverslips in DMEM supplemented with 10% FCS and 1 × penicillin/streptomycin in 5% CO₂ at 37 °C. Cells were washed with PBS and fixed in 4% formaldehyde, 0.1% Tween 20 in PBS. Cells were incubated for 15 min in TBS containing 2% BSA and 0.5% Tween 20 and for a further 60 min in TBS containing 2% BSA, 0.1% Tween 20, followed by incubation with primary and secondary antibodies at 23 °C. S-phase cells were identified by bromodeoxyuridine staining using the bromodeoxyuridine labelling and detection kit I (Roche).

RNAi

For RNA-mediated interference (RNAi) against UbcH10, cells were arrested with thymidine, released for 2 h and transfected with siRNA-oligos against UbcH10. After 4 h serum was added, and cells were again arrested with thymidine. Cells were washed, then released into fresh medium. Nocodazole was added after 4 h and samples were taken at times indicated. For RNAi against Emi1, Emi1/Cdh1 and Emi1/UbcH10, cells were arrested with thymidine, released for 1 h and treated with siRNA. Nocodazole was added with serum after 4 h. After a further 6 h, mitotic cells were collected by shake-off, then washed and released into fresh medium. All siRNAs were Smartpools from Dharmacon. Transfections were performed with Oligofectamine.

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No cold dust within the supernova remnant Cassiopeia A

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A large amount (about three solar masses) of cold (18 K) dust in the prototypical type II supernova remnant Cassiopeia A was recently reported¹. It was concluded that dust production in type II supernovae can explain how the large quantities ($\sim 10^8$ solar masses) of dust observed² in the most distant quasars could have been produced within only 700 million years after the Big Bang. Foreground clouds of interstellar material, however, complicate the interpretation of the earlier submillimetre observations of Cas A. Here we report far-infrared and molecular line observations that demonstrate that most of the detected submillimetre emission originates from interstellar dust in a molecular cloud complex located in the line of sight between the Earth and Cas A, and is therefore not associated with the remnant. The argument that type II supernovae produce copious amounts of dust is not supported by the case of Cas A, which previously appeared to provide the best evidence for this possibility.

It is well known that the Cas A supernova remnant is occulted by interstellar clouds of molecular gas³, as was demonstrated by the discovery of the OH molecule in absorption against it; this was the first radio detection of a molecule in interstellar space⁴. The visual extinction caused by dust associated with these clouds is probably the reason why only tentative historical records exist for the supernova outburst around AD 1680, although its proximity (3.4 kpc) would have made Cas A most probably the brightest stellar object in the sky⁵. The thermal emission of this foreground material should be easily detectable in submillimetre observations, given the high column densities of molecular gas ($N(\text{H}_2) > 10^{22} \text{ cm}^{-2}$) towards the supernova remnant⁶. Dunne *et al.*¹ indeed note “an area of diffuse emission to the west of the remnant”, which they removed, however, as being an instrumental artefact. Such extended emission was also noted independently by the observers who originally obtained the submillimetre data⁷ using the Submillimetre Common-User Bolometer Array (SCUBA)⁸.

To clarify the nature of the submillimetre emission, we have observed Cas A at 160 μm using the Multiband Imaging Photometer (MIPS)⁹ aboard the Spitzer Space Telescope. Not only does the spectral response of this band lie near the peak of the emission of cold interstellar dust, but the detectors are d.c.-coupled and operate in staring mode, so they respond well to extended emission. Our new measurements show an extended emission component (Fig. 1a and 2), distributed towards the southeast and west far beyond the forward shock that defines the outer boundary of the supernova remnant. The same large-scale emission was independently detected at 170 μm using the ISOPHOT¹⁰ Serendipity Survey¹¹, ISSS (Fig. 2).

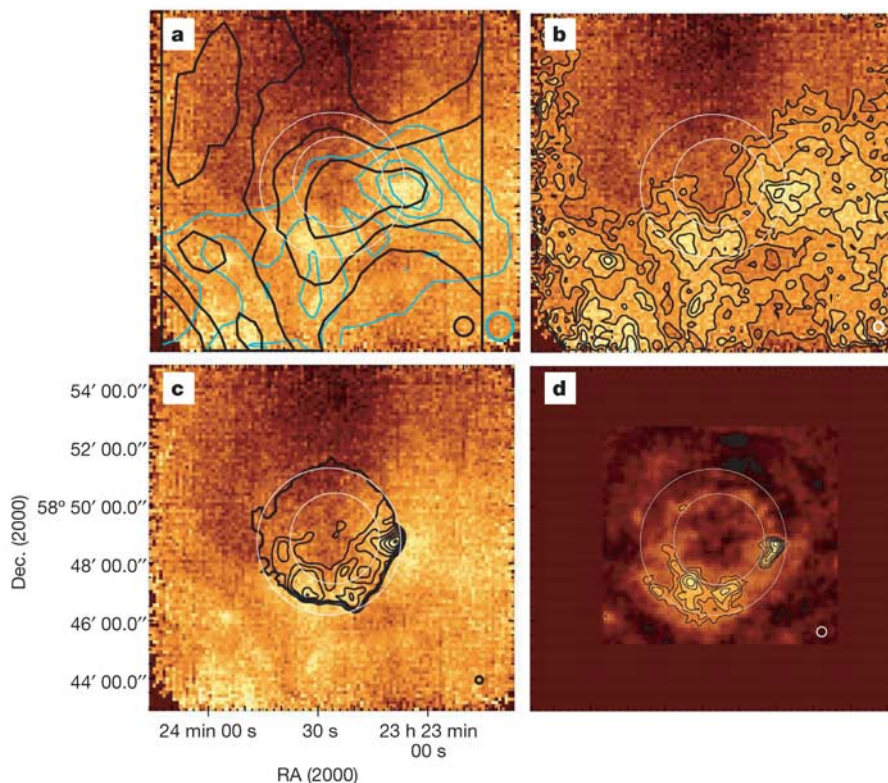


Figure 1 Continuum and molecular line emission from dust and gas. **a–c**, Nyquist-sampled SCUBA 850 μm images after removal of the synchrotron component by subtracting a 3.7 mm image²⁴ scaled to match the extrapolated¹² synchrotron flux of 35 Jy at 850 μm . The white circles indicate the position of the reverse and forward shocks estimated from X-ray observations. The total flux of integrated 850 μm dust emission within the forward shock is $16.0 \pm 2.4 \text{ Jy}$ ($1 \text{ Jy} = 10^{-26} \text{ W m}^{-2} \text{ Hz}^{-1}$). The contours overlaid are 160 μm surface brightness (black, increment 30 MJy sr^{-1}) and integrated intensity of $^{13}\text{CO } J = 1-0$ emission (blue, increment 2.5 K km s^{-1}) (**a**), 850 μm surface

brightness (increment 20 mJy per beam , starting at 95 mJy per beam) (**b**) and integrated optical depth of OH $2\Pi_{3/2} F = 2-2$ absorption (increment 0.1) (**c**). Beam sizes are indicated (circles at lower right). The OH and 3.7 mm observations have been convolved to match the $15''$ SCUBA beam and were sampled at the same positions. **d**, The baseline flattened and synchrotron subtracted 850 μm map from Dunne *et al.*¹ with the same contour levels as **b** for comparison. All panels show an area of $12' \times 12'$ centred at the remnant. Dec., declination; RA, right ascension.

In contrast, the chopping inherent in SCUBA data-taking suppresses such extended emission; low frequency noise in the bolometers can further complicate measurements of extended sources. Since different approaches to the data reduction can therefore yield different results about the extended background towards Cas A, we obtained the data used by Dunne *et al.*¹ from the SCUBA archive and re-reduced it. In our case, rather than forcing the baseline to zero, we determined it for each bolometer scan by subtracting the median level over the scan. The resulting submillimetre morphology after removal of the synchrotron emission (shown in Fig. 1a–c) is consistent with that observed at 160 μm .

Our submillimetre map confirms the presence of three knots of emission in the southeastern and western part of the remnant, which coincide with the emission detected by Dunne *et al.*¹ in their background subtracted map (compare Fig. 1d). We have not

reanalysed the SCUBA 450 μm data, but the image presented by Dunne *et al.* agrees well with their synchrotron-subtracted 850 μm image (Fig. 1d) and is dominated by the same knots. The 160 μm image also supports the presence of these knots, within the limitations of its lower angular resolution (a beam diameter of 40'' compared with 15'' for the 850 μm map). From the ratio of the flux densities at 160 and 850 μm we derive a dust temperature of 14 ± 2 K towards the dust clouds, a value commonly observed in dark clouds.

As shown in Fig. 1a, much of the 160 μm and 850 μm continuum emission coincides with the distribution of carbon monoxide¹² emission from a molecular cloud complex at a radial velocity (v_{LSR}) of -50 to -35 km s^{-1} with respect to the local standard of rest, which is kinematically associated with the Perseus spiral arm of our Galaxy. To extend the comparison of the dust emission with the shape of this molecular cloud to higher resolution, Fig. 1c shows the optically thin OH absorption¹³ by the molecular gas against the bright radio continuum background of Cas A. As the kinematics of the OH is similar to that of CO, it traces the same material. The correspondence of the OH absorption to the full resolution 850 μm emission image from Dunne *et al.*¹ (Fig. 1d) is extremely close, suggesting that much of the emission attributed by them to the Cas A supernova remnant instead arises in the foreground molecular material.

We can test quantitatively whether these molecular clouds could be responsible for the emission observed by Dunne *et al.*¹ by comparing the column densities of gas and dust towards the remnant. Whereas a gas-to-dust mass ratio of about 100–200 is expected for the interstellar medium, pristine dust synthesized by the supernova is not expected to be associated with molecular gas (or at most, in significantly smaller quantities). OH is preferred for this comparison because even ^{13}CO is optically thick³. The total column density of gas was determined from the observed OH line opacity using an abundance^{14,15} of $N(\text{OH})/N(\text{H}_2) = 8 \times 10^{-8}$ and

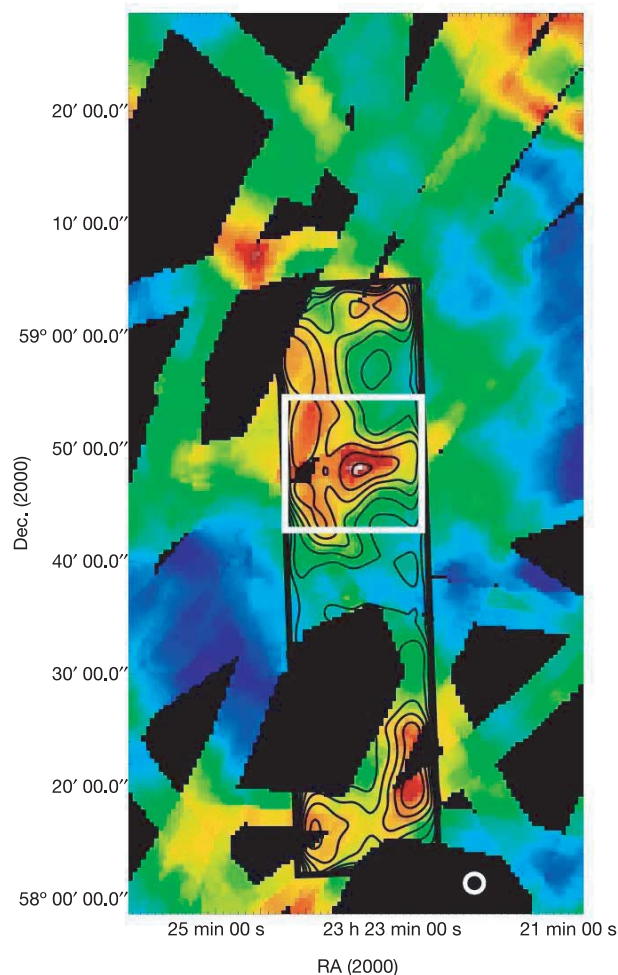


Figure 2 Large-scale far-infrared emission from dust. The data have been obtained using MIPS and ISOSS. The ISOPHOT 170 μm map in the background shows a $40' \times 80'$ large region centred on Cas A. Colours represent 170 μm surface brightness (range 140–300 MJy sr^{-1}). The MIPS 160 μm map (convolved to the 100'' ISOSS beam) is overlaid as contours. An issue with the MIPS data is that the integration ramps saturate on the bright background around Cas A. However, the individual amplifier reads at 8 s^{-1} are sent to the ground, and we found it possible to extract meaningful measurements from the first few reads before saturation. The quality of the data was confirmed by comparing integrated fluxes with those from ISO (the agreement was within the calibrational uncertainties of $\sim 20\%$) and through laboratory measurements that show that the recovery from saturation is quick without serious implications for the following integration. The larger field of the ISOSS map reveals the presence of dust emission extending considerably beyond the field shown in Fig. 1 (white box).

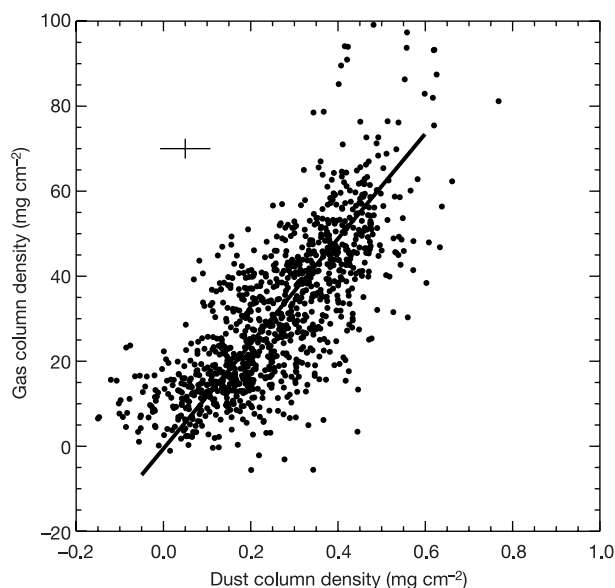


Figure 3 Gas-to-dust correlation. The column densities of dust and gas towards Cas A were determined point-by-point from the Nyquist-sampled 850 μm and OH maps within the outline shown in Fig. 1c (thick black line), where the radio brightness was high enough to measure accurately the strength of the OH absorption. The outer boundary of this area coincides with the forward shock of the remnant. The errors of the individual measurements (corresponding to the 1σ noise in the 850 μm and OH opacity maps) are indicated at upper left. The data can be fitted to a straight line with a slope of 120 ± 24 . The quoted 1σ uncertainty of the gas-to-dust mass ratio is a combination of the fitting uncertainty of the slope (which accounts for noise in the maps and temperature uncertainties) and the calibration errors ($\sim 10\%$ at 850 μm and $\sim 15\%$ for the OH measurements).

assuming that the excitation temperature equals the dust temperature of 14 K in these dense clouds. We derived the column density of dust from the optically thin 850 μm emission using a dust mass absorption coefficient¹⁶ of $\kappa_{850\mu\text{m}} = 1.8\text{ cm}^2\text{ g}^{-1}$. Both quantities are highly correlated, as shown in Fig. 3 (confidence level $P > 0.99$), corresponding to a gas-to-dust mass ratio of 120 ± 24 in full agreement with the canonical value for the Milky Way. To bound the uncertainties, we used the synchrotron subtracted and baseline flattened 850 μm map from Dunne *et al.*¹ to estimate a gas-to-dust ratio of 105 ± 21 . The good agreement with the value from our reduction of the same data shows that the result does not depend on the submillimetre data reduction procedure.

Assuming a distance of 3 kpc to the Perseus spiral arm clouds³, the total masses of dust and gas projected across the face of the remnant are $14 M_\odot$ and $1,700 M_\odot$ respectively, where $M_\odot$ is the solar mass. As the OH column density is seen in absorption against the radio emission of Cas A, the molecular material and dust accounting for most of the submillimetre emission must be located between the Earth and the remnant.

We can set an upper limit to the amount of cold dust in the supernova remnant using our 160 μm map, taken near the wavelength that should be most sensitive for its detection. After subtracting the interstellar foreground emission and the contribution from $0.003 M_\odot$ of warm (82 K) dust¹⁷ in Cas A, we derive an upper limit of $0.2 M_\odot$ of cold dust. We have assumed protosilicate grains, which are suggested to account for the bulk of warm dust in the remnant. As there is no evidence for large amounts of cold (18 K) dust within Cas A, exotic explanations for the submillimetre signal are unnecessary. (Such explanations include the non-standard dust opacities as proposed by Dunne *et al.*¹ or Dwek's proposal for metallic needles¹⁸.)

The dust yield of Cas A, the prototypical type II supernova, is therefore at least one order of magnitude lower than previously reported, weakening the argument that objects like it are the dominant source of interstellar dust. The presence of cold dust was recently also reported for the Kepler supernova remnant¹⁹. The generic type of this supernova is, however, controversial²⁰, with several lines of evidence pointing towards type Ia^{21,22}. In addition, the background/foreground emission towards the remnant is only slightly lower than for Cas A²³, raising the possibility that some of the submillimetre emission arises from material projected onto the remnant, as in Cas A. Therefore, by itself the Kepler remnant does not make a compelling case that large amounts of dust are created in type II supernova remnants. Because of the dearth of other well-established type II supernova remnants, and the tendency for them to lie close to the Galactic plane where infrared and submillimetre background/foreground emission is strong, it will be difficult to establish unequivocally the presence of large masses of cold dust in these objects. □

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Stellar encounters as the origin of distant Solar System objects in highly eccentric orbits

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The Kuiper belt¹ extends from the orbit of Neptune at 30 AU to an abrupt outer edge about 50 AU from the Sun². Beyond the edge is a sparse population of objects with large orbital eccentricities^{3,4}. Neptune shapes the dynamics of most Kuiper belt objects, but the recently discovered planet 2003 VB12 (Sedna⁵) has an eccentric orbit with a perihelion distance of 70 AU, far beyond Neptune's gravitational influence^{6–8}. Although influences from passing stars could have created the Kuiper belt's outer edge and could have scattered objects into large, eccentric orbits^{9,10}, no model currently explains the properties of Sedna. Here we show that a passing star probably scattered Sedna from the Kuiper belt into its observed orbit. The likelihood that a planet at 60–80 AU can be scattered into Sedna's orbit is about 50 per cent; this estimate depends critically on the geometry of the fly-by. Even more interesting is the ~ 10 per cent chance that Sedna was captured from the outer disk of the passing star. Most captures have very high inclination orbits; detection of such objects would confirm the presence of extrasolar planets in our own Solar System.

In the planetesimal theory, planets grow from mergers of smaller, slowly moving objects embedded in a gaseous, circumstellar disk surrounding a young star¹¹. For reasonable initial disk masses¹², this process yields 1,000-km radius planets with roughly circular orbits in

50–100 million years (Myr) at 70 AU (refs 13, 14). To scatter a 1,000-km planet into a Sedna-like orbit, a much larger planet must form at roughly the same distance. These more massive planets take at least a gigayear (Gyr) to form and should be rare. Despite extensive searches, none have been detected^{5,15}. Thus, *in situ* formation of a planet with Sedna's current properties seems unlikely.

A chance encounter between the Sun and another star is a more plausible explanation for the orbit of Sedna. Previous numerical simulations show that a fly-by of a solar-type star, with a distance of closest approach $q_f \approx 150$ –200 AU, scatters objects at 60–80 AU into very eccentric orbits and truncates the Kuiper belt at its observed edge^{9,10}. A more distant fly-by, with $q_f \approx 500$ –1,000 AU, lifts objects from Neptune-crossing orbits into Sedna-like orbits, leaving the rest of the Kuiper belt relatively unchanged¹⁶. Although other mechanisms for truncating the Kuiper belt are possible^{17–19}, they are inconsistent with the apparent formation of 1,000-km planets in the larger disks observed around several nearby stars^{20,21}. Here we improve on previous fly-by calculations and derive an initial orbital distribution of Kuiper belt objects from a detailed planet-formation calculation^{22,23}. We then use a complete set of *N*-body simulations to provide the first estimates of the likelihood that a close encounter would have produced the observed edge to the Kuiper belt and planets in Sedna-like orbits.

The discovery of Sedna also prompted us to consider an exciting new possibility: captures from the Kuiper belt of the passing star. Like most stars, the Sun probably formed in a star cluster and experienced a close encounter with another cluster member²⁴. Encounters with field stars are unlikely²⁵. Cluster lifetimes of 100 Myr to 1 Gyr (ref. 24) allow a solar-type star to produce 1,000-km objects as far out as 100 AU. Thus, both the Sun and the passing star probably had extended planetary disks at the start of their encounter. By analogy with galaxy collisions^{26,27}, the two stars can exchange a significant amount of outlying material during a fly-by, depending on the geometry of the encounter and the properties

of the planetary systems. Here we adopt a cluster age of 30–200 Myr for the encounter, and use planet formation and *N*-body simulations to provide the first assessment of the probability that a close pass could yield the observed edge to the Kuiper belt and the capture of an extrasolar planet into a Sedna-like orbit.

Consistent with formation in a star cluster with a velocity dispersion²⁸ of 1 km s^{-1} , we assume that the star and the Sun have equal mass and orbit as a marginally bound pair. Modest differences in starting conditions have no qualitative impact on our results. During the fly-by, tidal shear dramatically increases orbital eccentricities in the disk at large heliocentric distances; objects closer to the Sun are unperturbed. Simulations that produce a sharp edge in the Kuiper belt yield a correlation between q_f and i_f , the inclination of the fly-by trajectory relative to the invariable plane of the Solar System. For low-inclination orbits, where the passing star corotates with the disk, q_f must exceed 120 AU to avoid disrupting the Kuiper belt. Counter-rotating fly-bys with $i_f = 90^\circ$ – 180° require smaller q_f values to produce the observed edge (Fig. 1). The simulations suggest i_f (in degrees) $\approx 275 - 1.6q_f$. For a specific $q_f = 90$ –160 AU, this expression yields i_f to an accuracy of $\approx 10\%$ for corotating encounters and i_f to $\approx 30\%$ for counter-rotating orbits, where tidal shear is more broadly distributed in the disk and the edge is less sharp. In both cases, post-fly-by collisional grinding removes additional material from the Kuiper belt and accentuates the outer edge. Detailed collision models^{22,23} suggest that grinding and shear reduce the mass by 98% to 99.9%, which is close to observational estimates²⁹.

We may further constrain the passing star's trajectory by assuming that Sedna is indigenous to the Solar System. Simulations then identify the fly-by configurations that scatter objects into Sedna-like orbits, defined to have modest inclination ($i < 30^\circ$), and large eccentricity and perihelion distance ($e > 0.5$ and $q > 50$ AU). Figure 2 shows results for two encounters where planets are scattered from nearly circular orbits at 80 AU into Sedna-like orbits, a corotating fly-by with $q_f = 160$ AU and $i_f = 23^\circ$, and a counter-rotating fly-by with $q_f = 90$ AU and $i_f = 172^\circ$. Both fly-bys scatter $\sim 10\%$ of the objects from an annulus at 80 ± 2.5 AU into high-eccentricity orbits. After 50–200 Myr, our coagulation calculations produce 5–25 planets with radii of 500–2,000 km at 80 ± 2.5 AU. Thus, a fly-by that makes the current edge to the Kuiper belt also produces at least one indigenous object in a Sedna-like orbit. Roughly 95% of these 'successful' fly-bys are corotating encounters. The chance of a Sedna-like orbit is fairly independent of the starting distance or the initial orbital eccentricity. Roughly 2% of the 40–200 large planets formed in circular orbits at 60–80 AU scatter into a Sedna-like orbit. We derive similar results for the 50–500 planets with $e > 0.5$ and $q > 35$ AU in a 'scattered disk' (Fig. 3), suggesting that formation of a Sedna-like orbit from a scattered disk is two to three times more likely than formation from planets in initially circular orbits. Assuming both paths produce Sedna-like orbits, we predict 3–30 Sedna-like objects compared to the 30–100 estimated from the detection of a single Sedna at 90 AU.

Surprisingly, the probability that captured planets have Sedna-like orbits is also significant. When the trajectory of the Sun and the disk of the passing star corotate, the Sun captures up to a third of the extrasolar objects initially in orbit at distances of 60–80 AU from the passing star (Fig. 2). This 'capture efficiency' depends on q_f and the angle i_f' between the rotational axes of the two disks. The capture efficiency is 10% to 30% for $q_f \leq 120$ AU and $i_f' \leq 80^\circ$, and falls to $\sim 5\%$ for $q_f \approx 160$ AU and $i_f' \leq 45^\circ$. For fly-bys with many captures, counter-rotating fly-bys yield more Sedna-like objects than corotating fly-bys. For a random ensemble of fly-bys that produce the observed edge of the Kuiper belt, the probability of a capture into a Sedna-like orbit is $\sim 5\%$.

The probability estimates for indigenous and captured planets with Sedna-like orbits assume a fly-by that produces the observed outer edge of the Kuiper belt. Simple estimates of the evolution of

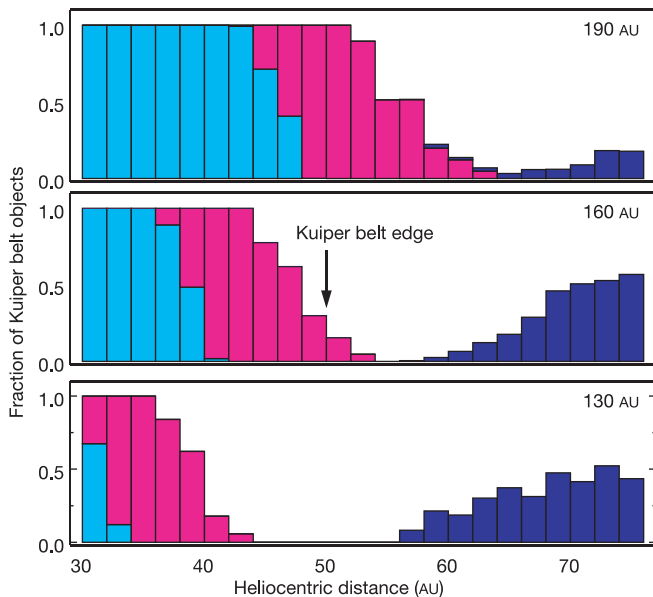


Figure 1 Stirring of the eccentricities of planets by the close pass of a Sun-like star. The star is on a marginally bound orbit corotating with the Sun's disk, with an orbital inclination of $i = 23^\circ$ and argument of perihelion $\omega = 212^\circ$. Each panel lists the distance of closest approach. The histograms show the frequency of the final eccentricity, e_f , for 20,000 particles initially in circular orbits at 40–80 AU around the Sun. Magenta histograms show the fraction of orbits with $e_f < 0.04$; cyan histograms show the fraction of orbits with $e_f < 0.2$; blue histograms show the fraction of orbits with Sedna-like orbits ($e_f > 0.5$). The 160-AU encounter places the edge of the Kuiper belt where it is actually observed, as indicated by the arrow in the middle panel.

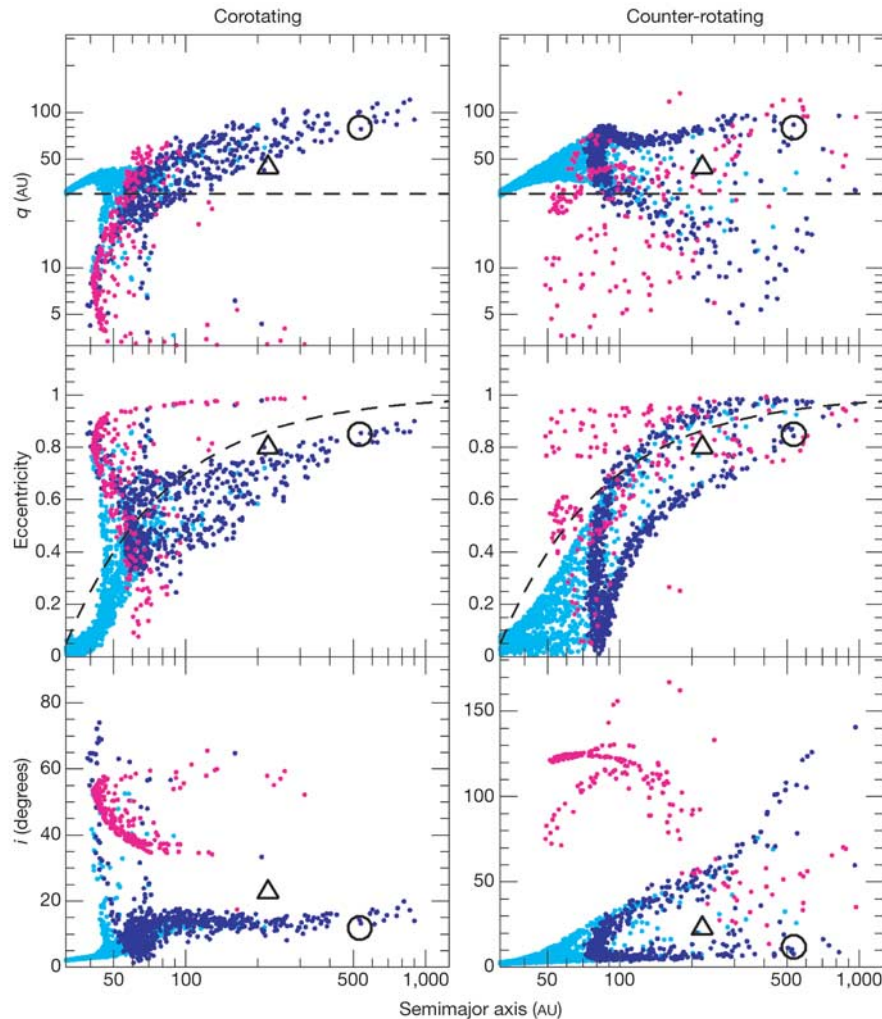


Figure 2 Orbital elements of planets, formed *in situ* in the planetary disk, after the fly-by of a one-solar-mass star as a function of final heliocentric distance. The planets have an initial probability p of formation in 1-AU bins at 40–80 AU, derived from detailed planet-formation calculations; $p \propto a^{-1}$, where a is the semimajor axis. The left panels show a marginally bound, corotating fly-by ($d_{\text{close}} = 160$ AU, $i = 23^\circ$ and $\omega = 212^\circ$). The right panels show a marginally bound counter-rotating fly-by ($d_{\text{close}} = 90$ AU, $i = 172^\circ$ degrees, and $\omega = 130^\circ$). Large black circles represent the orbital elements of Sedna.

Large black triangles represent the orbital elements of 2000 CR₁₀₅. In the upper panels, objects below the dashed lines cross the orbit of Neptune; in the middle panels, objects above the dashed lines cross the orbit of Neptune. Cyan points indicate planetary orbits initially distributed between 30–80 AU (Fig. 1), with initial eccentricity $e_0 = 0.02$ and initial inclination $i_0 = 0.5^\circ$. Blue points indicate orbits initially at 80 ± 2.5 AU, with $e_0 = 0.05$ and $i_0 = 1^\circ$. Magenta points indicate objects captured by the Sun from the disk of the passing star. Each simulation consisted of 1,000 particles.

star clusters indicate a reasonable probability, $\sim 15\%$, for a stellar encounter within 160 AU during the first 1 Gyr of the solar lifetime²⁴. Because the Sun is heavy enough to remain in the cluster as the cluster evaporates, 30% to 50% of the encounters probably occur during the first 100 Myr of the solar lifetime. Thus the total probability of producing at least one object with a Sedna-like orbit in the Solar System is reasonably large, $\sim 5\%$ to 10% for an indigenous object and $\sim 1\%$ for a captured object. These probabilities are significant compared to the chance of finding a Solar System like our own around any solar-type star, $\sim 1\%$ or less²⁴.

The simulations demonstrate that fly-bys leave unique signatures on the dynamics of the outer Solar System. Corotating fly-bys produce a single population with orbital inclination $i \approx 10^\circ$ at 100–500 AU; counter-rotating fly-bys produce a cold population with $i \leq 10^\circ$ and a hotter population with $i \geq 30^\circ$ (Fig. 2). Fly-bys with an initial scattered disk of objects at 60–80 AU yield broader, but still distinct, distributions in inclination (Fig. 3). The two inclination populations of counter-rotating fly-bys qualitatively resemble the two observed populations of Kuiper belt objects at 40–50 AU (ref. 30). Systematic searches for high-inclination objects in the outer Solar System can distinguish between corotating and counter-rotating fly-bys and can estimate the relative fraction of objects in a

scattered disk at the time of the fly-by.

Orbits of individual objects also test the models. Some fly-bys produce objects with orbital elements similar to 2000 CR₁₀₅, a $\sim 1,000$ -km planet with $q = 44$ AU, $e = 0.8$ and $i = 22^\circ$. Although 2000 CR₁₀₅ is closer to Neptune than Sedna, the known planets probably could not have scattered 2000 CR₁₀₅ into its present orbit^{7,16}. The corotating fly-by in Fig. 2 cannot produce orbits with the large range of inclination observed in Sedna and 2000 CR₁₀₅, but counter-rotating fly-bys yield a broad range of orbits that encompass the observations. Both types of fly-by generate orbits similar to those of 2000 CR₁₀₅ and Sedna from a scattered disk (Fig. 3). Long-term simulations suggest that interactions with Neptune broaden the inclination distributions of corotating fly-bys, forming objects with orbital elements closer to both 2000 CR₁₀₅ and Sedna. From these simulations, we estimate that 2000 CR₁₀₅ is two to three times more likely to be a captured planet than Sedna. Because our calculations are the only known way to produce high-inclination objects, searches at high ecliptic latitude provide the best test of this mechanism for Sedna formation. Detection of objects with $i \geq 40^\circ$ would clinch the case for the presence of extrasolar planets in the outer Solar System. $\square$

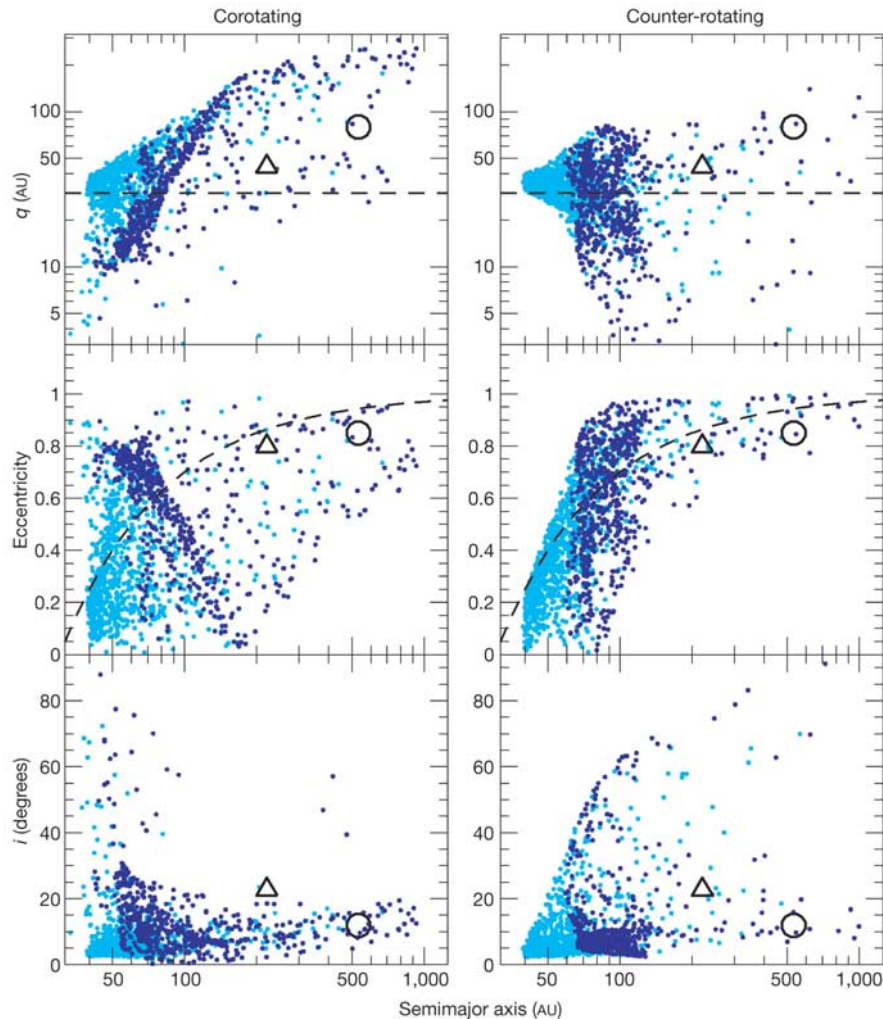


Figure 3 Orbital elements of planets, initially in a 'scattered disk', after a fly-by, as described in Fig. 2. The planets are placed initially at 40–80 AU with $p \propto a^{-1}$, as in Fig. 2, $q = 35$ AU, and $i_0 \leq 5^\circ$. Cyan points indicate planetary orbits initially distributed between 60–80 AU with a perihelion distance of 35 AU. Blue points indicate orbits initially at 80 ± 2.5 AU, also with a perihelion distance of 35 AU. The objects in the figure end up

with a broader range of orbital elements than those in Fig. 2. Although a subset of particles is consistent with both the orbits of Sedna and 2000 CR₁₀₅, the distributions are broader, reducing the correlations between the orbital elements of scattered objects and the good agreement between the observations and the models evident for the *in situ* formation shown in Fig. 2.

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Realization of quantum error correction

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Scalable quantum computation¹ and communication require error control to protect quantum information against unavoidable noise. Quantum error correction^{2,3} protects information stored in two-level quantum systems (qubits) by rectifying errors with operations conditioned on the measurement outcomes. Error-correction protocols have been implemented in nuclear magnetic resonance experiments^{4–6}, but the inherent limitations of this technique⁷ prevent its application to quantum information processing. Here we experimentally demonstrate quantum error correction using three beryllium atomic-ion qubits confined to a linear, multi-zone trap. An encoded one-qubit state is protected against spin-flip errors by means of a three-qubit quantum error-correcting code. A primary ion qubit is prepared in an initial state, which is then encoded into an entangled state of three physical qubits (the primary and two ancilla qubits). Errors are induced simultaneously in all qubits at various rates. The encoded state is decoded back to the primary ion one-qubit state, making error information available on the ancilla ions, which are separated from the primary ion and measured. Finally, the primary qubit state is corrected on the basis of the ancilla measurement outcome. We verify error correction by comparing the corrected final state to the uncorrected state and to the initial state. In principle, the approach enables a quantum state to be maintained by means of repeated error correction, an important step towards scalable fault-tolerant quantum computation using trapped ions.

Error-correcting codes that utilize entanglement to rectify unknown errors in qubits are an important ingredient for large-scale quantum information processing^{1–3} (QIP). Owing to the fragility of quantum states, the presence of noise during both storage and entangling operations diminishes any gain achieved through the use of QIP without error correction. For example, the realization of long-distance quantum cryptography requires protection from the effects of noise on communication channels⁸. In addition, quantum error correction will be necessary for applications of quantum computing such as efficient factorization of large numbers^{1,9}. It is notable that any of an infinite set of qubit errors can be corrected through quantum error correction by means of a finite set of unitary operations conditioned on measurement.

Although there has been extensive theoretical research in the area of quantum error correction, experimental work has been limited to protocol process verifications by means of liquid-state nuclear magnetic resonance. These experiments^{4–6} showed an increase in state fidelity after performing the unitary operations of an error-correction protocol, but using techniques known not to scale efficiently with the number of qubits⁷. Furthermore, the ancilla cannot be reset in these experiments, whereas the experiment we describe provides this capability. In principle, the protocol demonstrated here can be repeated with the same qubits as many times as required by a particular quantum algorithm.

We describe the implementation of a quantum error-correcting code (QECC) using three physical qubits, here denoted as the primary qubit and ancilla 1 and 2, to encode and protect one logical qubit from spin-flip errors (a π rotation around the x axis, in this case). Each qubit comprises two hyperfine states of a $^9\text{Be}^+$ ion and can be coherently manipulated by means of laser pulses^{10,11}. We implement the QECC by (1) preparing the state the primary qubit, (2) encoding this state into the logical state of all three qubits through use of an entangling operation, (3) applying an error rotation (that induces spin-flips upon measurement) to all three qubits, (4) decoding the logical state to the primary qubit, (5) measuring the state of the ancilla, and finally (6) applying correction operations to the primary qubit dependent upon the ancilla measurement outcome. The error correction is performed deterministically in every experiment. The results indicate that such methods, coupled with other recent experimental advances^{11–14}, may lead to scalable QIP in a system of trapped ions.

In contrast to the standard repetition code^{2,3,15}, the QECC described and implemented here (see Fig. 1) can not be obtained by application of the superposition principle to a classical error-correction code. It is a stabilizer code¹⁵ with stabilizer group generators $\{ZZX, ZXZ\}$. These generators are tensor products of Pauli operators ($X = \sigma_x$, $Z = \sigma_z$) operating on the qubits 1, 2 and 3. By calculation of the commutation relations of the generators with various error sets, it can be seen that as an error-detecting code, the QECC presented here detects not only spin-flips on any qubit but also a spin-flip or phase-flip on qubits 2 and 3. As an error-correction code, it will correct a spin-flip on any of the three qubits (the case for this work), but it can instead be used to correct a spin-flip on qubit 1 and a spin-flip or phase-flip on qubit 2 or qubit 3, for example. Another possible implementation can correct a spin-flip on qubit 1 and a phase-flip on either of the other two qubits.

In the experiment, we use $^9\text{Be}^+$ ions confined to the axis of a multi-zone linear radio frequency Paul trap¹¹ similar to that described in ref. 16. The qubits comprise the electronic ground-state hyperfine levels $|F=1, m_F=-1\rangle$ and $|F=2, m_F=-2\rangle$ (denoted as $|\uparrow\rangle$ and $|\downarrow\rangle$ respectively, by analogy to the states of a spin- $\frac{1}{2}$ particle). Here F is the total angular momentum and m_F is its projection along the quantization axis. The qubits are entangled with a three-qubit phase gate utilizing the ions' quantized axial vibrational modes¹⁷ (see Methods section and Fig. 2b). Rotations

$$R(\theta, \phi) = \cos \frac{\theta}{2} I - i \sin \frac{\theta}{2} \cos \phi \sigma_x - i \sin \frac{\theta}{2} \sin \phi \sigma_y \quad (1)$$

are realized with two-photon stimulated-Raman transitions^{10,18} implemented by two laser beams having a relative frequency detuning equal to the qubit transition frequency. Here σ_x and σ_y are the usual Pauli operators, I is the identity operator, θ is the rotation angle, and ϕ is the angle of the rotation axis in the x - y plane. Rotations around an axis A by an angle θ will be denoted A_θ , for example, $X_{\pi/2} = R(\pi/2, 0)$, although for rotations around an axis by π we will omit the angle subscript, for example, $X = R(\pi, 0)$.

Measurement is accomplished through projection of the state of each qubit using state-dependent resonance fluorescence (an ion in the $|\downarrow\rangle$ state fluoresces, whereas an ion in the $|\uparrow\rangle$ state does not). The ions can be transported from one zone of the trap to another

through concerted variation of the potentials on segmented control electrodes of the trap, and individual ion detections can be performed separately^{11,19}.

Ion preparation before each implementation of the QECC protocol consists of Doppler cooling, Raman sideband cooling of all three axial modes of vibration to the ground state, and optical pumping of the ions to the $|\downarrow\downarrow\downarrow\rangle$ state^{10,20}. Each experiment also requires the initialization of the primary physical qubit to a state $|\psi_0\rangle_P = \alpha|\uparrow\rangle_P + \beta|\downarrow\rangle_P$ with the ancillae initialized to the state $|\downarrow\downarrow\rangle_A$. This is accomplished by momentarily increasing the spacings between the three ions and then applying a rotation that affects the ions differently owing to their respective positions in the laser beam intensity profile (see Fig. 2a). This operation requires only one laser pulse¹⁰. The state of the primary qubit is then encoded in the state of all three qubits (see Methods section). In the following discussion we assume perfect entangling operations.

After encoding, we apply an 'error' θ_e , a rotation X_{θ_e} , to all qubits by means of a stimulated-Raman transition with all ions illuminated equally (see equation (1) and top part of Fig. 2a). With respect to later measurement, this error induces a spin-flip on each physical qubit with probability $p(\theta_e) = \sin^2(\theta_e/2)$. The state is then decoded using the inverse of the operation used for encoding. The decoding effects a transformation such that afterwards, the four possible states of the ancillae in the measurement basis (the error syndromes) depend on the type of error that has occurred: the state $|\uparrow\uparrow\uparrow\rangle_A$ corresponds to no error having occurred, the state $|\uparrow\uparrow\downarrow\rangle_A$ corresponds to the first ancilla having flipped, the state $|\uparrow\downarrow\uparrow\rangle_A$ corresponds to the second ancilla having flipped, and the state $|\downarrow\downarrow\downarrow\rangle_A$ corresponds to the primary qubit having flipped. For at most one qubit flipped, the state of the primary qubit before a correction operation is applied is shown in Table 1.

After the decoding operation, the ions are spatially separated (see Fig. 2c), and a measurement of the state of the ancillae is performed.

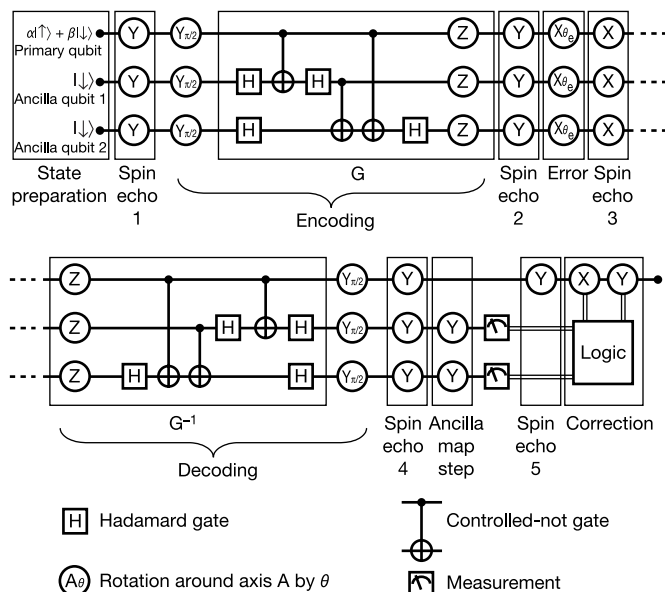


Figure 1 Quantum circuit for the quantum error-correction protocol described and implemented in this work as one would compose it of single-bit rotations, Hadamard gates, and controlled-not gates¹⁵. Double lines denote classical information. The entangling operation $G(=G^{-1})$ is diagonal in the measurement basis and is defined in the Methods section. The operation of G requires only one collective pulse on all three ions as implemented in this work. The spin echo refocusing operations and ancilla mapping are discussed in the Methods section. For rotations around an axis by π we omit the angle subscript, for example, $X = R(\pi, 0)$ (see equation 1). The Hadamard and controlled-not operations that make up the entangling operation G are equivalent to three controlled-phase rotations between permuted pairs of qubits; these operations could be substituted for the ones shown, depending on the system-dependent entangling gates selected.

The ions are then moved so that only the primary qubit ion is addressed. Depending on the ancillae measurement outcome, a correction operation (X , Y or I , see Table 1) is applied to the primary qubit. This qubit is then analysed to determine the effectiveness of the protocol. After initial cooling and preparation of the state $|\downarrow\downarrow\downarrow\rangle$, each experiment requires approximately 4 ms to perform.

In principle, this QECC works perfectly only when at most one of the three qubits undergoes a spin-flip error. The probability of more than one qubit flipping is given by:

$$P_{2\text{or}3}(\theta_e) = p^3(\theta_e) + 3p^2(\theta_e)[1 - p(\theta_e)] \quad (2)$$

Because of this, most input states cannot be corrected to all orders in the error θ_e , though they can be corrected such that an improvement in the fidelity over the uncorrected case is attainable for small errors. The fidelity of the corrected final state (as derivable from the action of the code in Fig. 1) as a function of the error will be:

$$F(\theta_e) = 1 - |\alpha|^2|\beta|^2(2 - 3\cos\theta_e + \cos^3\theta_e) \quad (3)$$

$$\approx 1 - \frac{3}{4}|\alpha|^2|\beta|^2\theta_e^4 + O(\theta_e^6)$$

The infidelity in this case is quadratic in θ_e^2 , whereas it is linear in θ_e^2 for the uncorrected state. The fidelity reaches a maximum value of 1 for the cases where $|\alpha|^2|\beta|^2 = 0$. The input states $|\downarrow\downarrow\rangle_P$ (where $\alpha = 0, |\beta| = 1$) and $|\uparrow\uparrow\rangle_P$ (where $|\alpha| = 1, \beta = 0$) can therefore be

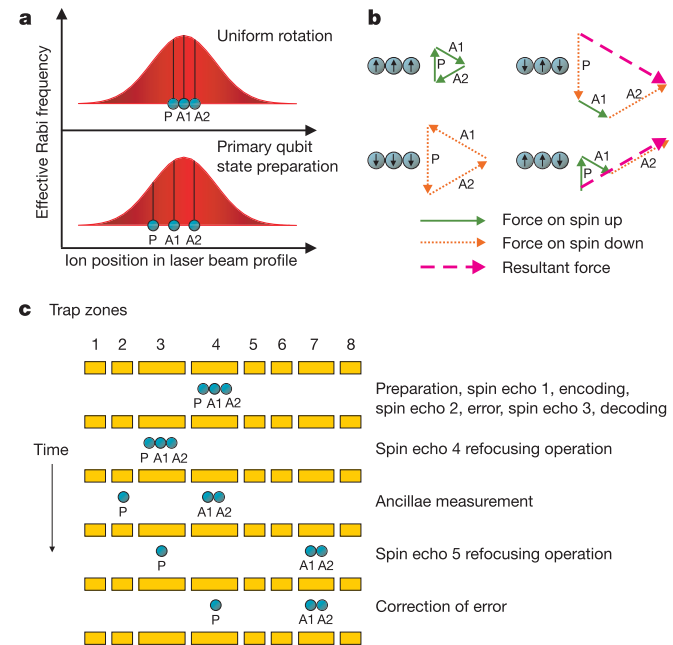


Figure 2 Schematic representation of experimental techniques. **a**, State preparation method. The upper panel depicts ion locations in the laser beam profile for global rotations. In the lower panel, the ions' spacings are increased such that their relative Rabi frequencies for rotations are different (the spacings between the ions relative to the laser beam width are exaggerated in this diagram). By moving the ions such that the primary qubit (labelled P) is at a location of lower radiation intensity, and hence has a lower Rabi frequency than the ancillae A1 and A2 (which are illuminated with equal intensity), a rotation of $2n\pi$ (n an integer; for this experiment $n = 2$) can be performed on the ancillae while any chosen rotation dependent upon the relative Rabi frequencies can be performed on the primary qubit¹⁰. **b**, Optical-dipole forces (directions indicate relative phase) on ions during the entangling operation G for different internal states. The state-dependent force¹⁸ ($F_1 = -2F_2$) on each ion leads to accumulation of phase if the centre-of-mass vibrational mode is excited, that is, when the internal states of the ions are different, as in the two diagrams on the right. **c**, Transportation of ions during the error-correction protocol. The three ions' positions are shown as a function of time. Qubit operations are performed on ions in trapping regions 3 and 4, and ion separations are performed in region 3.

Table 1 Syndromes and corrections for the quantum error-correcting code

Primary qubit before correction	Error	Ancillary syndrome	Correction operation
$\beta \uparrow\rangle_P + \alpha \downarrow\rangle_P$	No error	$ \uparrow\uparrow\rangle_A$	X
$\alpha \uparrow\rangle_P + \beta \downarrow\rangle_P$	Ancilla 1 flipped	$ \uparrow\downarrow\rangle_A$	I
$\alpha \downarrow\rangle_P + \beta \uparrow\rangle_P$	Ancilla 2 flipped	$ \downarrow\uparrow\rangle_A$	I
$\beta \uparrow\rangle_P - \alpha \downarrow\rangle_P$	Primary qubit flipped	$ \downarrow\downarrow\rangle_A$	Y

The first column shows the state of the primary qubit before a correction is applied. The fourth column shows the correction required to recover the primary qubit initial state $|\psi_0\rangle_P = \alpha|\uparrow\rangle_P + \beta|\downarrow\rangle_P$.

corrected to all orders in θ_e for any error X_{θ_e} with this protocol.

To isolate the basic behaviour of the protocol from the technical errors present in the coherent operations required for its implementation, we apply the complete protocol (Fig. 1) with and without application of the error-correction operations and compare the final state fidelities. Figure 3a shows the results for the input state $|\downarrow\rangle_P$. The data are consistent with the theoretical prediction that the state should be corrected for any error X_{θ_e} by this QECC. The curves have non-zero infidelity for zero error angle θ_e owing to infidelity present in the operations of the protocol.

Figure 3b and c shows similar data for the initial states $\sqrt{0.10}|\uparrow\rangle_P - i\sqrt{0.90}|\downarrow\rangle_P$ and $\sqrt{0.22}|\uparrow\rangle_P - i\sqrt{0.78}|\downarrow\rangle_P$ respectively. From equation (3), it can be seen that as the input state gets closer to the equator of the Bloch sphere ($|\alpha| \approx |\beta|$), the QECC is expected to perform worse for larger errors. However, the infidelity of the

corrected state should grow only quadratically in θ_e^2 for small errors, as opposed to a linear growth for the uncorrected state. This behaviour can be observed in the data, which in all cases show an improvement over the uncorrected state. Data for a full range of input states are not presented, as input states with larger values of $|\alpha|$ required larger ion spacings and lower axial confining potentials than were practical during preparation of the primary qubit state; these configurations were temporally unstable in the current apparatus. It should be noted, however, that these experimental limitations to state preparation do not affect the QECC protocol.

We also plot the observed rate of the ancillary syndromes for a particular input state in Fig. 3d to verify that our QECC protocol is correctly detecting errors. The rate $\Gamma(|\uparrow\uparrow\rangle_A)$ of the syndrome corresponding to no error and the sum of the rates $\Gamma(|\uparrow\downarrow\rangle_A) + \Gamma(|\downarrow\uparrow\rangle_A) + \Gamma(|\downarrow\downarrow\rangle_A)$ of the syndromes corresponding to one error are plotted separately. The rates of the syndromes vary in the predicted manner for increasing error, up to an offset due to imperfections in the operations that make up the protocol.

Although an improvement in fidelity over an uncorrected encoded qubit was observed, the QECC protocol as implemented here induced more infidelity for small errors than would be acquired if an unencoded qubit had been initialized and then been subject only to the applied error. It should be noted, however, that for errors

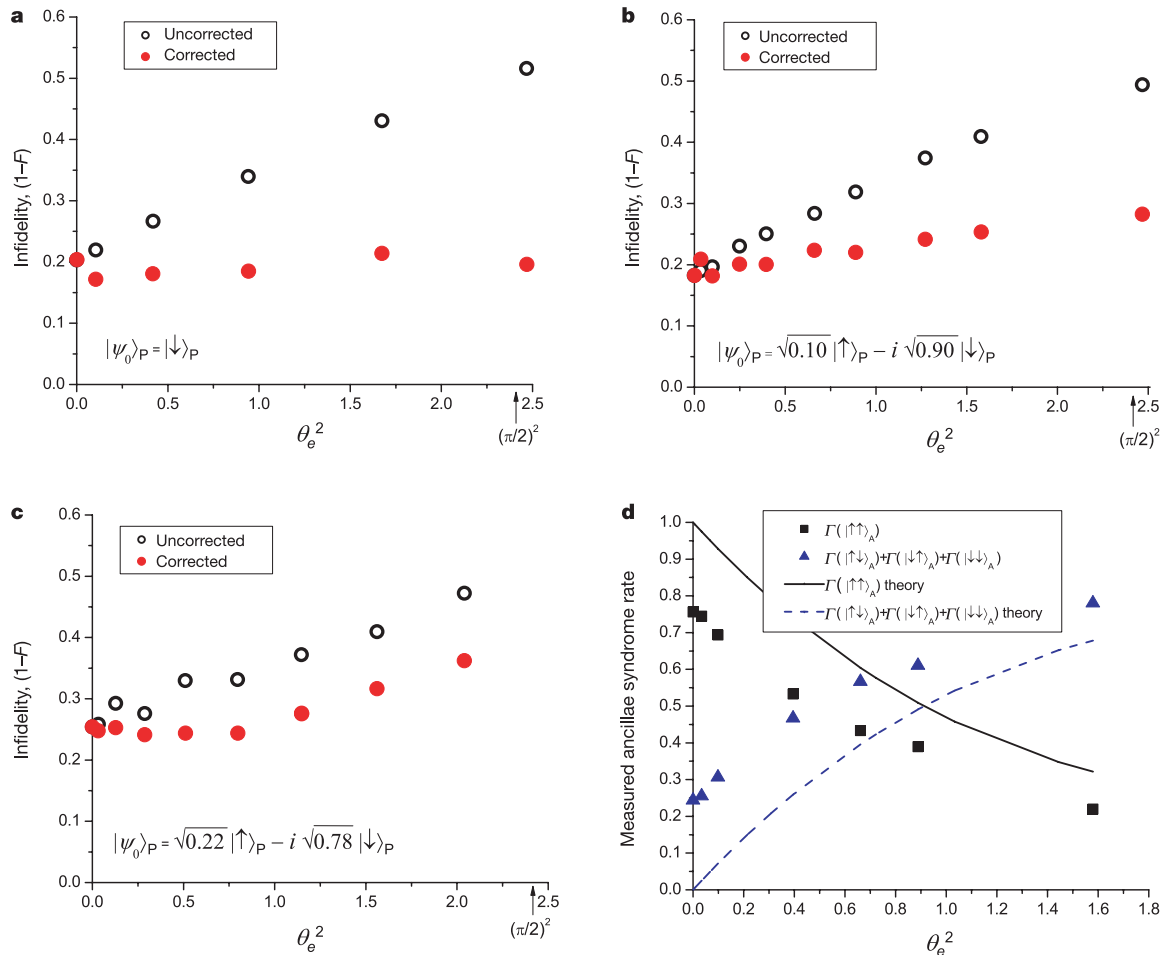


Figure 3 Results of quantum error correction protocol. Recovered state infidelity plotted versus the square of the applied error for corrected and uncorrected cases for three initial states (**a–c**) and rate of syndrome measurements of ancillae (**d**). One-standard-deviation errors are approximately the size of the symbols. **a**, The initial state is $|\psi_0\rangle_P = |\downarrow\rangle_P$. **b**, The initial state is $|\psi_0\rangle_P = \sqrt{0.10}|\uparrow\rangle_P - i\sqrt{0.90}|\downarrow\rangle_P$. **c**, The initial state is $|\psi_0\rangle_P = \sqrt{0.22}|\uparrow\rangle_P - i\sqrt{0.78}|\downarrow\rangle_P$.

$\sqrt{0.22}|\uparrow\rangle_P - i\sqrt{0.78}|\downarrow\rangle_P$. **d**, Rate of syndrome measurements of ancillae versus square of error angle. The rate $\Gamma(m)$ is the measured probability of obtaining the measurement outcome m . The data are off-set from the theoretical curves because of imperfect gate operations. The initial state is $|\psi_0\rangle_P = \sqrt{0.10}|\uparrow\rangle_P - i\sqrt{0.90}|\downarrow\rangle_P$ for these data.

larger than $\theta_e \approx 1$ radian, the corrected state had higher fidelity than an unencoded qubit undergoing only an applied error for all states investigated, and the logical state was genuinely protected with this protocol. The degradation observed during the execution of the QECC is due in large part to the fidelity of the encoding and decoding gates ($\sim 90\%$), and all operations must be improved to achieve fault-tolerance. In spite of these technical difficulties, the current experiment shows the viability of the error-correction protocol and demonstrates the feasibility of quantum error correction in a scalable system in which the ancillae can be reset. With improvements in fidelity, the execution of the QECC can be made a useful part of more complex quantum algorithms. $\square$

Methods

Encoding and decoding

To encode the logical state with the QECC, we implement a three-ion entangling operation, an extension of those described previously^{12,17}:

$$G(s_1, s_2, s_3) = G^{-1}(s_1, s_2, s_3) = \begin{cases} 1 & \text{if } s_1 = s_2 = s_3 \\ -1 & \text{otherwise} \end{cases} \quad (4)$$

Here $s_i \in \{ \uparrow, \downarrow \}$ is the spin state of the i th ion. This operation is diagonal in the measurement basis and is equivalent to that depicted by G in Fig. 1, as can be verified by explicit calculation. A 'walking wave' polarization interference pattern is set up at the ions' location using two perpendicular beams of radiation whose wavevector difference is parallel to the trap axis and whose difference frequency is detuned by a small amount δ ($\approx 2\pi \times 70$ kHz) from the frequency ω_{COM} ($= 2\pi \times 3.7$ MHz) of the centre-of-mass (COM) axial vibrational mode in the trap^{17,18}. The inter-ion spacing is adjusted relative to the beams' interference pattern so that if the phase of the resulting oscillating optical-dipole force at frequency $\omega_{\text{COM}} - \delta$ at the primary ion is ϕ_p , the phases at the ancilla ions are $\phi_{A1} = \phi_p + \frac{2\pi}{3}$ and $\phi_{A2} = \phi_p + \frac{4\pi}{3}$. The axial COM mode will be (off-resonantly) excited only if there is a net force on the ions, which is the case if the ions are in different internal states, that is, for all states except $|\uparrow\uparrow\uparrow\rangle$ and $|\downarrow\downarrow\downarrow\rangle$ (see Fig. 2b). As the vibrational mode is excited, the ions move along a closed path in phase space (if the radiation is applied for a time equal to $2\pi/\delta$), and the states acquire a phase proportional to the phase-space area enclosed by this path. All states except those for which $s_1 = s_2 = s_3$ acquire the same phase (adjusted to be π), as the magnitude of the net force on the ions is equal and non-zero. The application of a global rotation $Y_{\pi/2}$ followed by this entangling gate implements the 'Encoding' section of Fig. 1. The application of an error is followed by a re-application of the three-ion entangling gate G and a final global rotation $Y_{\pi/2}$; this implements the 'Decoding' section of Fig. 1.

Refocusing and auxiliary operations

To simplify the syndrome determination when the ancillae are measured together, the protocol is designed such that an X operation is required to return the protected qubit to its initial state if no error has occurred, while no operation (the identity) is required if either one of the ancillae is flipped, and a Y operation is required if the primary bit is flipped. The protocol was implemented in this fashion through the tailoring of the phases of two spin echo refocusing pulses^{11,16,17} that are required between encoding and decoding (spin echoes 2 and 3 in Fig. 1) to counteract qubit dephasing caused by fluctuations in the local magnetic field from experiment to experiment. The time required for ion transportation in the trap necessitates additional spin-echo operations after decoding and before correction (spin echoes 4 and 5). To reduce experimental measurement uncertainty, an ancilla mapping step is performed to associate the outcome that is expected most often (no error) with the ancilla state $|\uparrow\uparrow\uparrow\rangle_A$, the state most easily distinguishable from the others when the ancillae are measured together.

After the correction operation is applied to the primary qubit, we apply a rotation X_{θ_p} to this qubit before measurement to characterize the effectiveness of the protocol. The angle θ_p is varied over many experiments such that we produce a sinusoidal trace of the probability of measuring the primary qubit to be in the state $|\downarrow\rangle_p$ as it is rotated around the Bloch-sphere axis around which the error was applied. This allows a fit to a sinusoidal curve and hence precise measurement of the final state fidelity of the primary qubit.

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Nonlinear optics in the extreme ultraviolet

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Nonlinear responses to an optical field are universal in nature but have been difficult to observe in the extreme ultraviolet (XUV) and soft X-ray regions owing to a lack of coherent intense light sources. High harmonic generation is a well-known nonlinear optical phenomenon^{1,2} and is now drawing much attention in attosecond pulse generation^{3–6}. For the application of high harmonics to nonlinear optics in the XUV and soft X-ray regime, optical pulses should have both large pulse energy and short pulse duration to achieve a high optical electric field. Here we show the generation of intense isolated pulses from a single harmonic (photon energy 27.9 eV) by using a sub-10-femtosecond blue laser pulse, producing a large dipole moment at the relatively low (ninth) harmonic order nonadiabatically^{7,8}. The XUV pulses with pulse durations of 950 attoseconds and 1.3 femtoseconds were characterized by an autocorrelation technique, based on two-photon above-threshold ionization⁹ of helium atoms. Because of the small cross-section for above-threshold ionization¹⁰, such an autocorrelation measurement of XUV pulses with photon energy larger than the ionization energy of helium has not hitherto been demonstrated^{6,11–13}. The technique can be extended to the characterization of higher harmonics at shorter wavelengths.

The two-photon above-threshold ionization (ATI) of helium atoms is a successive absorption of an additional photon beyond the one required for ionization of helium atoms. The cross-section of two-photon ATI is an order of magnitude smaller than conven-

tional two-photon ionization¹⁰. When a nonlinear optical process is observed, it becomes possible to characterize the pulses by the intensity autocorrelation, which is the standard diagnostic technique in the visible region¹⁴. In the autocorrelation measurement, the intensity of nonlinear optical signal is recorded as a function of the delay time between two replicas of a laser pulse. The autocorrelation measurement is therefore the simplest type of pump-probe experiments immediately extendable to femtosecond and attosecond nonlinear optical spectroscopy. In comparison with synchrotron radiation (SR), which also covers the XUV and soft X-ray regions, high harmonic pulses have temporal coherence and short pulse duration—that is, high peak intensity. Thus, high harmonics are expected to open the new field of XUV nonlinear optics.

Previous work motivated us to generate and characterize high harmonic pulses experimentally in the expectation of generating intense attosecond pulses (Fig. 1). We focus on the ninth harmonic generated by the sub-10-fs blue laser pulse (photon energy 3.1 eV) in a multi-cycle regime, in contrast with previously reported attosecond pulse generation in a few-cycle regime from the coherently superimposed broadband continuum in the cut-off region^{4,5}. From nonadiabatic viewpoints, in which the slowly varying envelope approximation is not valid, Christov *et al.* simulated the 51st high harmonic generation of a 25-fs Ti:sapphire laser¹⁵. The 51st pulse lasts for about three optical cycles with ionization of the interacting gas medium. A driving laser with a shorter pulse duration and shorter wavelength is expected to generate a shorter pulse. Together with the nonadiabatic effect, the dipole moment of lower order induced by the blue laser is larger than that induced by a Ti:sapphire laser⁷. An enhancement of the pulse energy is therefore expected. From the adiabatic viewpoints¹⁶, a ninth-harmonic pulse of a 3.1-eV laser pulse was simulated by using a zero-range potential model¹⁷. The ninth-harmonic pulse driven by an 8-fs pulse evolves steeply with the highly nonlinear response of the dipole moment in the rising edge of a driving pulse. When the optical electric field becomes high enough to ionize the interacting atoms¹⁸, high harmonic generation is shut off because of the small dipole moment of the ions. In this case, a driving laser with a shorter pulse duration is also preferred.

With the foregoing as a basis, modifications could be made by taking account of the spatial intensity distribution in the driving laser beam and the propagation of harmonics. For example, harmonic pulses would be generated at different times at different positions, but the phase-matching effect would not preserve the difference¹⁹. However, two-dimensional or three-dimensional simulations of high harmonic generation indicate that the harmonic pulses still retain the features of the single atom in the cut-off

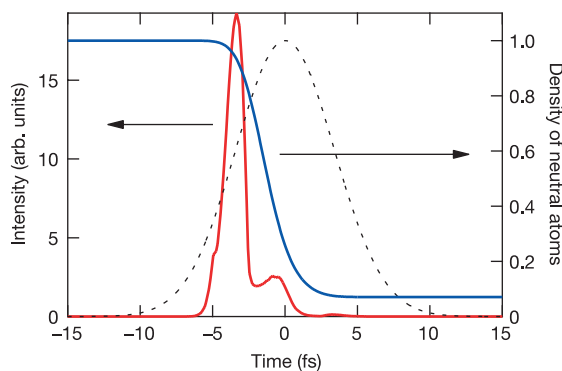


Figure 1 High harmonic pulse generation in the adiabatic picture. The red line is the ninth harmonic pulse of the 8-fs driving pulse with a peak intensity of $5.5 \times 10^{14} \text{ W cm}^{-2}$ (dashed line). The blue line is the density of the neutral Ar atoms radiating high harmonics calculated by using a tunnelling ionization theory¹⁸. The generation of high harmonics ceases with the ionization of neutral atoms.

region^{8,15}. The autocorrelation measurement will make it possible to investigate these effects experimentally.

Blue laser pulses lasting 8.3 fs and 12 fs were generated by the frequency conversion of a Ti:sapphire laser (photon energy 1.55 eV) as driving laser pulses^{20,21}. Figure 2 shows the experimental setup for the autocorrelation measurement. Two optically delayed replicas of a blue laser pulse were focused into an argon gas jet to generate the ninth high harmonic. The gas jet operating at 1 kHz was placed 4 mm after the focus and there was no spatial overlap between two beams at high harmonic generation, where the peak intensity was $3.9 \times 10^{14} \text{ W cm}^{-2}$. The fundamental and lower-order harmonics were eliminated by an aluminium filter. The ninth-harmonic pulses were focused into helium gas by an Sc/Si multilayer spherical mirror with a focal length of 5 cm. The total pulse energy on the target was 2 nJ. The ejected photoelectrons were collected and energy-resolved by a magnetic bottle photoelectron spectrometer, ensuring a high collection efficiency of 50%. Figure 2b is the photoelectron spectrum of two-photon ATI. The observed highest photoelectrons ejected by a one-photon process were from the 11th harmonic absorption of Ar atoms and the peak by two-photon ATI was well separated from the other peaks, ensuring the assignment and the two-photon process. An autocorrelation trace was recorded by measuring the electron number at each optical delay.

The autocorrelation traces obtained by using 8.3-fs and 12-fs laser pulses are shown in Fig. 3a and Fig. 3b, respectively. Two electric fields superimpose coherently at a delay time of 0 fs, leading to the enhancement of two-photon ATI. The ratio of the peak to the

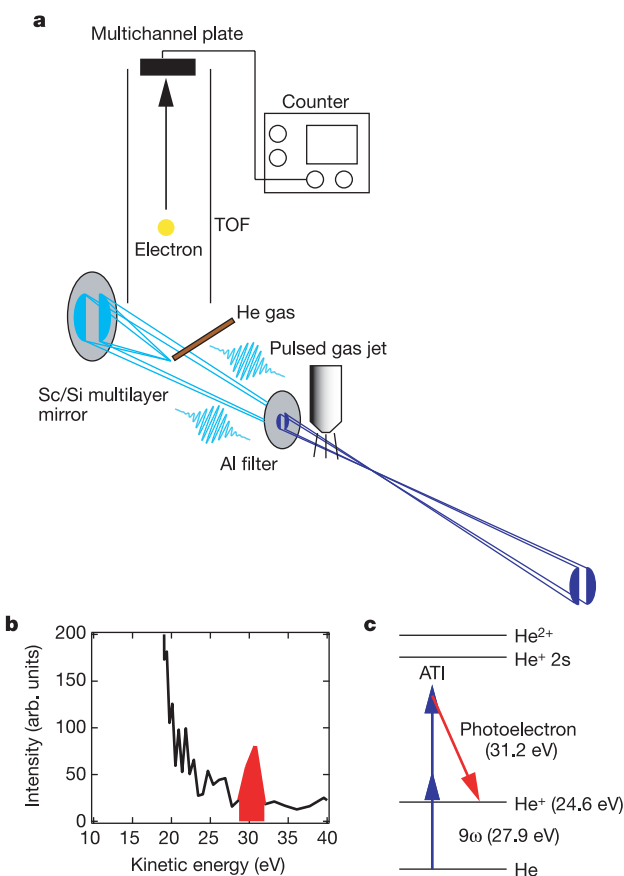


Figure 2 Two-photon above-threshold-ionization (ATI) autocorrelator. **a**, Experimental setup for the autocorrelation measurement of the ninth harmonic (9ω) of the blue laser. To improve the spectral resolution of the photoelectron spectrometer, an electrostatic field was applied to the time-of-flight (TOF) tube and the photoelectrons were decelerated inside the tube. **b**, The photoelectron spectrum. **c**, Diagram of the two-photon ATI process. The area in red in **b** indicates the photoelectrons ejected from He atoms by the process shown in **c**.

background was almost 3:1, in good agreement with the ideal case of the autocorrelation measurement, indicating that the peak is not the coherent spike. To estimate the pulse durations of the ninth harmonics, the autocorrelation traces were fitted to gaussian functions; the results are shown by the red lines in Figs 3a and 3b. The corresponding full widths at half maximum of the autocorrelation traces were 1.3 ± 0.1 and 1.8 ± 0.1 fs, resulting in pulse durations of 950 ± 90 as and 1.3 ± 0.1 fs, respectively.

In the 950-as pulse, however, bumps appeared around the main peak and the gaussian function does not seem to be appropriate to describe the pulse shape. To check the validity of the experimental results, the spectra of the ninth harmonic (Fig. 3c) were Fourier-transformed with an assumption of a flat phase in the frequency domain, and the autocorrelation functions were then calculated. The results are shown by the blue lines in Fig 3a, b. Both the autocorrelation trace of the 1.3-fs pulse and that of 8.3-fs pulse are reproduced well. The bumps are therefore attributable to the spectrum shape. Consequently, no other pulses were observed within the scanned time range of 20 fs, showing the isolated single

pulses. This is an advantage of the autocorrelation measurement over electron streaking, in which the measurable time range is limited by one-half of the optical cycle²². In future this method will be extended to frequency-resolved optical gating (FROG)²³ by improving the spectral resolution to characterize the harmonic pulses completely.

The spectra of the ninth harmonic reflect the spectra of the Ti:sapphire and blue lasers. To increase the spectrum width, the gain around 800 nm was reduced by inserting an etalon in a regenerative amplifier of the Ti:sapphire laser system, resulting in a spectrum with two peaks²⁴. This type of spectrum gives a relatively short pulse duration, although side bumps appear. Two peaks in the harmonic spectra are made prominent by the nonlinear process.

For further pulse shortening to the attosecond timescale with the use of a multi-cycle driving laser, one experimental approach is to generate higher-order harmonics; the lower-order harmonics rise earlier than the higher-order harmonics, resulting in a longer pulse duration. Higher-order harmonics would therefore be adequate for attosecond pulse generation, and a driving laser with shorter pulse duration is also required. However, it would be difficult to shorten the temporal duration below the half cycle of the driving laser (650 as). The feature of this scheme is a peak intensity high enough to induce nonlinear phenomena.

Finally, we estimate the photoelectron numbers created by the two-photon ATI. The photoelectron yield Y from the interaction volume V ($= 3.1 \times 10^{-9} \text{ cm}^3$) with an atomic density n ($= 10^{11} \text{ cm}^{-3}$) is given by $Y = \sigma^{(2)} F^2 \tau n V$, where $\sigma^{(2)}$ is the cross-section of two-photon ATI, F is the photon flux, and τ is the pulse duration. In the present experimental conditions, F and τ were $7.8 \times 10^{30} \text{ photons s}^{-1} \text{ cm}^{-2}$ and 1 fs, respectively, and $\sigma^{(2)}$ was set to 10^{-52} cm^2 from ref. 10. Consequently, Y is estimated to be 1.6×10^{-3} electrons per pulse. The observed yield was 1.0×10^{-3} electrons per pulse, taking account of the collection and quantum efficiencies, which is consistent with the estimation. □

Methods

Driving laser

Blue laser pulses were generated by broadband frequency doubling of the Ti:sapphire laser pulses to obtain shorter pulses than originally generated²¹. To broaden the bandwidth of the laser pulse, broadband frequency conversion was realized by spatial dispersion of each spectrum component of the Ti:sapphire laser pulse so that it was phase-matched²⁰. The pulse energies of the blue laser pulses were 1.3 mJ at a repetition rate of 1 kHz. The pulse durations were controlled by changing the spectral bandwidth of the Ti:sapphire laser system. The optical system for spectrum dispersion was modified to employ a telescope configuration for a large beam diameter of 2 cm. If this was not done, spatial pulse-front tilt and phase mismatch were so large that high harmonics were not generated. Spatial coherence of the driving laser is also important for generating two identical harmonic pulses. The pulses were characterized by self-diffraction frequency-resolved optical gating and were found to be Fourier-transform limited.

Autocorrelation measurement

In the present experiment, two harmonic pulses were generated from two beams produced by spatially dividing a blue laser beam into two. This is different from conventional autocorrelation measurements in the visible range, in which a beam splitter is used. However, the original driving laser beam had high spatial coherence, ensured by interfering the inverted image with the original one, so the two harmonic pulses naturally retained coherence. There still remained the possibility of generating different pulses because of inequality in beam division. However, because the energy difference was less than a few per cent, the harmonic pulses generated were almost identical.

Another method of producing two identical harmonic pulses has been shown⁶, involving the division of a harmonic beam spatially into two by a split XUV mirror after harmonic generation. However, in this case too the two pulses are not always identical. Because of the intensity distribution within a driving laser beam, harmonic pulses are generated at different times. So, unequal spatial division of the harmonic beam leads to the production of two different harmonic pulses even when a split XUV mirror is used.

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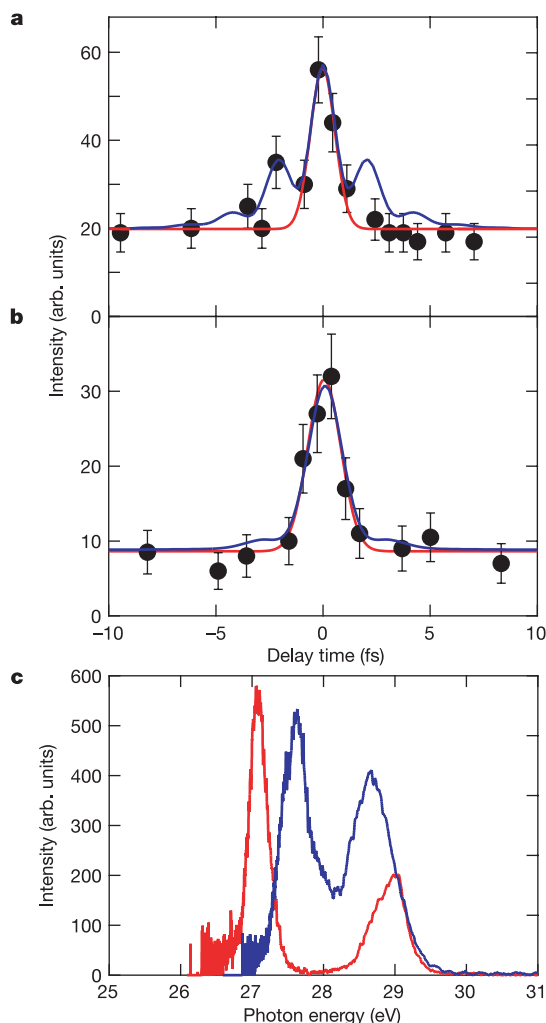


Figure 3 Autocorrelation traces and the spectra of the ninth harmonic of the blue laser. **a, b**, Autocorrelation traces of the ninth harmonic pulses generated by using 8.3-fs (**a**) and 12-fs (**b**) blue lasers. The error bars show the standard deviation of each data point. The full widths at half maximum of the autocorrelation traces obtained by the least-squared fit to the gaussian functions were 1.3 ± 0.1 and 1.8 ± 0.1 fs, resulting in pulse durations of 950 ± 90 as and 1.3 ± 0.1 fs, respectively. The lines in red show the fitting results. The lines in blue are the calculated autocorrelation traces from the spectra (**c**) of the ninth harmonic. **c**, The spectra of the ninth harmonic pulses generated by using 12-fs and 8.3-fs pulses are shown by the blue and red lines, respectively.

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Large fluctuations in speed on Greenland's Jakobshavn Isbræ glacier

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It is important to understand recent changes in the velocity of Greenland glaciers because the mass balance of the Greenland Ice Sheet is partly determined by the flow rates of these outlets. Jakobshavn Isbræ is Greenland's largest outlet glacier¹, draining about 6.5 per cent of the ice-sheet area, and it has been surveyed repeatedly since 1991 (ref. 2). Here we use remote sensing data to measure the velocity of Jakobshavn Isbræ between 1992 and 2003. We detect large variability of the velocity over time, including a slowing down from 6,700 m yr⁻¹ in 1985 to 5,700 m yr⁻¹ in 1992,

and a subsequent speeding up to 9,400 m yr⁻¹ by 2000 and 12,600 m yr⁻¹ in 2003. These changes are consistent with earlier evidence for thickening of the glacier in the early 1990s and rapid thinning thereafter³. Our observations indicate that fast-flowing glaciers can significantly alter ice discharge at sub-decadal time-scales, with at least a potential to respond rapidly to a changing climate.

Because of its contribution to the Greenland Ice Sheet's mass balance, an airborne laser altimeter has surveyed the Jakobshavn Isbræ glacier repeatedly since 1991 (ref. 2). When many other glaciers were thinning, these surveys revealed that Jakobshavn Isbræ thickened substantially from 1991 to 1997 (ref. 4) at elevations less than 1,100 m. After 1997, the glacier began thinning rapidly with peak rates of ~15 m yr⁻¹ (ref. 3).

We used interferometric synthetic aperture radar (InSAR) to measure Jakobshavn Isbræ's velocity in 1992, 1994, 1995 and 2000 (ref. 5). We also applied feature-tracking methods to Landsat-image pairs to determine velocity in 2001, 2002 and 2003. Earlier velocity measurements were made using airborne imaging from the summer of 1985 (ref. 6) and by tracking crevasse displacements in laser-altimeter surveys from 1997 (ref. 7).

Figure 1 shows the 1992 and 2000 speeds, illustrating a large speed-up by 2000 that extends over much of the fast-moving region. This increase is also visible in plots of the glacier's centreline speed (Fig. 2). Inland of ~40 km, there is no appreciable change over the 18-yr period. Closer to the ice front, the 2000-to-2003 speeds increase steadily over time relative to the fairly stable mid-1990s. From 1985 to 1992, however, the glacier slowed just before observed thickening³.

Figure 3 shows speeds on the fastest-moving (>5,000 m yr⁻¹) areas averaged at over 20 locations corresponding to the 1985 measurements⁶ (Fig. 1, purple symbols) and at several hundred locations corresponding to measurements⁷ made in 1997 (Fig. 1, orange line). The 1985 mean speed was 6,700 m yr⁻¹, which in 1992 decreased to 5,700 m yr⁻¹ and remained nearly constant until 1997. Between May 1997 and October 2000, the glacier speed reached 9,400 m yr⁻¹ and flowed at about this rate in May 2001. Over the next two months, the glacier speed increased to 10,000 m yr⁻¹. By summer 2002, the speed had increased to 11,900 m yr⁻¹ and then to 12,600 m yr⁻¹ in spring 2003, which is the last period for which we have velocities.

The mid-1990s slow interval corresponds well with the 1991-to-1997 measured thickening³. The 1985 calving rate was ~26.5 km³ yr⁻¹ (ref. 8), so the 1,000 m yr⁻¹ drop in speed by 1992 suggests a discharge reduction of ~4 km³ yr⁻¹. Most of the slowdown occurred over the region moving faster than 1,000 m yr⁻¹. If we assume thickening was confined to this fast-moving area, then we estimate an average thickening of ~1 m yr⁻¹, which is consistent with observed rates³. The 1985-to-2003 speed-up implies a near-doubling in discharge to 50 km³ yr⁻¹. Excluding the former ice tongue's area, this implies an average thinning of ~6 m yr⁻¹ over the fast-moving region, which is also consistent with observations³. Thus, the speed variations are sufficient to explain the observed elevation changes as dynamic thickening and thinning³.

Meltwater input to the bed seasonally affects nearby inland ice speed⁹, but these fluctuations are far smaller than the changes we observe. Although we have only coarse intra-annual sampling, our results suggest a trend of increasing speed starting after 1997, rather than a seasonal pattern of summer speed-up and winter slow-down⁹. The speed-up appears to persist through the winter, so it seems unlikely that increased surface-meltwater input to the bed directly caused the acceleration. Earlier observations that found no seasonal variation on Jakobshavn Isbræ^{6,10} support this hypothesis, as does our analysis of 2001–2002 spring and summer Landsat data.

From 1851 to 1953 the calving front retreated by ~26 km but stabilized¹¹, and it remained within a 2.5-km-long calving zone from 1962 to the 1990s¹². In October 2000, this pattern changed when a progressive retreat began that resulted in nearly complete disintegration of the ice shelf by May 2003.

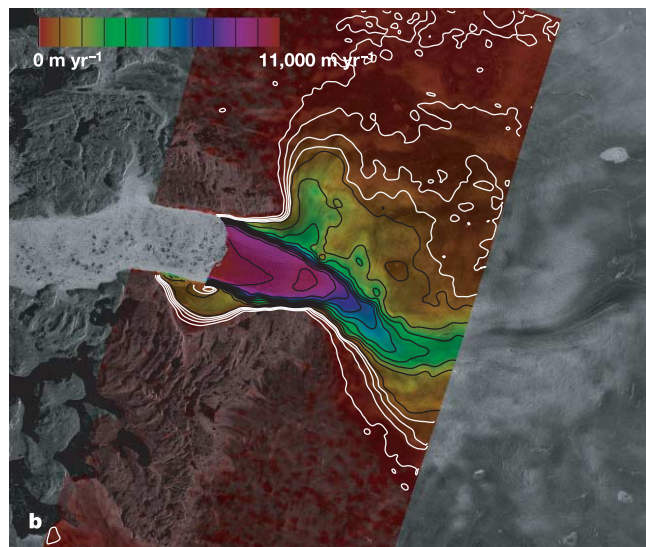
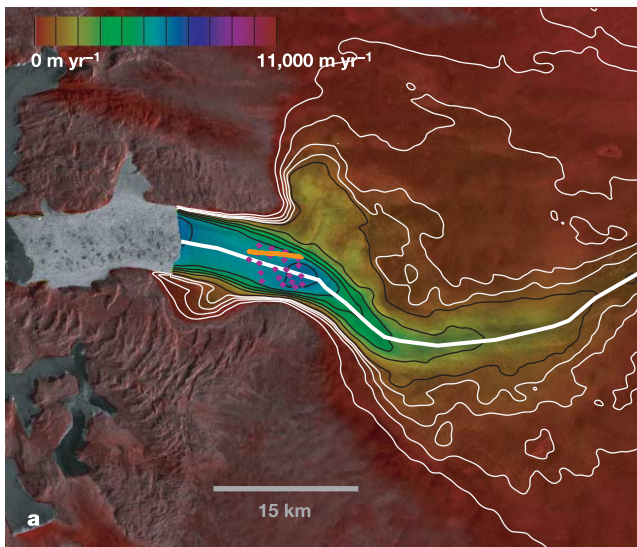


Figure 1 Ice-flow speed as colour over SAR amplitude imagery of Jakobshavn Isbræ in February 1992 (**a**) and October 2000 (**b**). In addition to colour, speed is contoured with thin black lines at $1,000 \text{ m yr}^{-1}$ intervals and with thin white lines at 200, 400, 600 and

800 m yr^{-1} . The thick white line shows the location of the profile plotted in Fig. 2. The locations of velocity measurements made in 1997 (orange line) and 1985 (purple symbols) are also shown.

The calving front's multi-decadal stability has been attributed to resistance from the fjord walls and pinning points that may have yielded a restraining "back-stress" on the floating ice with an influence extending above the grounding zone^{1,13}. Several of the speed increases coincide with losses of sections of the ice tongue as it broke up. Satellite images also indicate that several large rifts began developing in 2000 along the fjord's northern wall, which may have reduced lateral resistance. Thus, back-stress reduction from the ice tongue's disintegration may have caused or contributed to the speed-up. This is consistent with observations elsewhere of acceleration following ice shelf loss^{14,15}.

If the ice tongue's weakening and retreat caused the speed-up, then it is not clear what initiated the retreat after a period of multi-decadal stability¹². At nearby Egedesminde, the 1998–2001 melt intensity was abnormally high³ and passive microwave data indicate a high melt extent in 2002. Calving rates are higher in summer¹²,

indicating a sensitivity to temperature and melt^{13,16,17}. Thus, increased calving may have forced the ice tongue to retreat beyond its stable position to initiate the acceleration.

Jakobshavn Isbræ's acceleration and near-doubling of ice discharge is noteworthy because this single glacier has increased the rate of sea-level rise by $\sim 0.06 \text{ mm yr}^{-1}$, or $\sim 4\%$ of the 20th-century rate¹⁸. If the retreat of the ice tongue caused the acceleration, then similar losses of floating ice tongues since the Little Ice Age¹¹ may explain¹³ the current rapid thinning observed for many of Greenland's outlet glaciers^{4,19}. This speed-up is the most striking of several ice-stream and outlet-glacier speed-ups and slow-downs observed, with just over a decade of spaceborne InSAR measurements^{20–23}. Collectively, these observations indicate that fast-flowing glaciers can significantly alter ice-sheet discharge at sub-decadal timescales and that their response to climate change has at least the potential to be rapid. This argues both for further investigation of ice-sheet mass balance and improved incorporation of fast flow features into ice-sheet models, many of which predict response times on the centennial-to-millennial scale²⁴. □

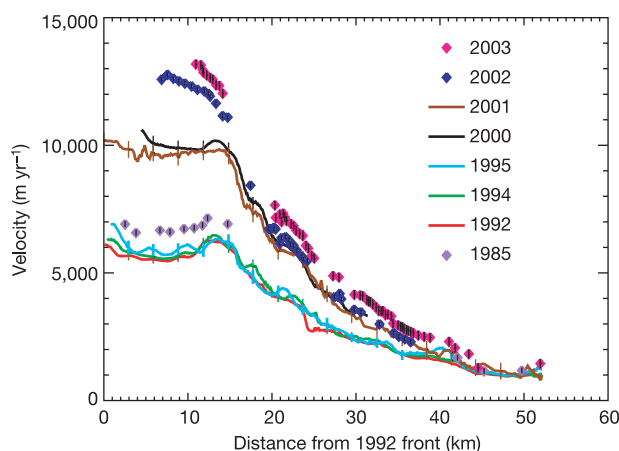


Figure 2 Profiles of speckle-tracked (see Methods), Landsat, airborne feature-tracked speed from along the line shown in Fig. 1a and as a function of distance from the approximate position of the 1992 calving front. One-sigma error bars are shown at several-kilometre intervals. The 1985 (ref. 6), 2002 and 2003 data sets were sparsely sampled, so only individual point measurements are plotted. The data were acquired over the periods from February to March 1992, December 1993 to March 1994, November 1995, October to November 2000, May 2001, July to September 2002, and March to May 2003.

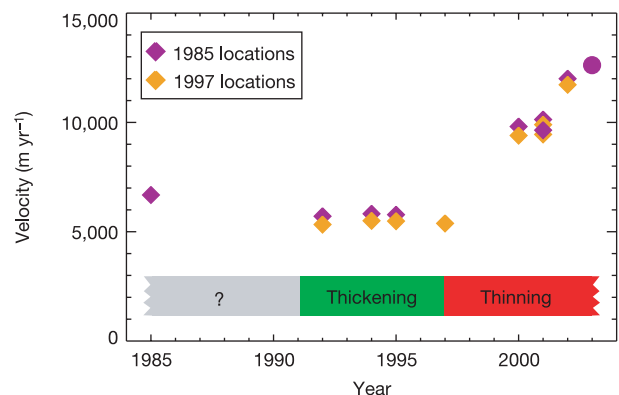


Figure 3 Average speeds versus time for measurements made at original 1985 and 1997 airborne survey locations (same-colour symbols in Fig. 1). For 2001, the slower two points are from a 4–20 May pair, whereas the faster points are from a 20 May to 7 July pair. Laser altimeter surveys determined the periods of thickening and thinning³. Minor speed differences for 1985 and 1997 locations reflect the different areas that were sampled. Because much of the floating ice tongue was missing in 2003, the speed for this year (circle) is an average of the points on the remaining ice.

Methods

A set of speckle-tracking algorithms⁵ was used to determine the 1992, 1994, 1995 and 2000 velocities from 1–24-day image pairs. Speckle tracking uses the displacements of the correlated speckle patterns in pairs of SAR images to derive ice motion estimates. Individual errors were up to a few hundred metres per year (see Fig. 2), but errors on averages (for example, Fig. 3) are below 100 m yr⁻¹. We did not tide-correct the speckle-tracked data, so there are biases on the floating ice that do not spatially average out. To assess this error, we estimated velocity for five 1992 InSAR pairs, each with different tidal errors. The standard deviation for these estimates was 69 m yr⁻¹. Our 1992 and 1994 estimates are temporal averages of multiple (2 to 5) same-year pairs, which further reduces this error. The 2001 through 2003 estimates were derived using the IMCORR²⁵ feature-tracking software applied to 16-to-64-day Landsat image pairs. Established methods²⁶ were applied to passive microwave data to determine the 2002 melt extent.

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Human contribution to the European heatwave of 2003

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The summer of 2003 was probably the hottest in Europe since at latest AD 1500^{1–4}, and unusually large numbers of heat-related deaths were reported in France, Germany and Italy⁵. It is an ill-posed question whether the 2003 heatwave was caused, in a simple deterministic sense, by a modification of the external influences on climate—for example, increasing concentrations of greenhouse gases in the atmosphere—because almost any such weather event might have occurred by chance in an unmodified climate. However, it is possible to estimate by how much human activities may have increased the risk of the occurrence of such a heatwave^{6–8}. Here we use this conceptual framework to estimate the contribution of human-induced increases in atmospheric concentrations of greenhouse gases and other pollutants to the risk of the occurrence of unusually high mean summer temperatures throughout a large region of continental Europe. Using a threshold for mean summer temperature that was exceeded in 2003, but in no other year since the start of the instrumental record in 1851, we estimate it is very likely (confidence level >90%)⁹ that human influence has at least doubled the risk of a heatwave exceeding this threshold magnitude.

Temperatures near the Earth's surface are rising globally¹⁰, and evidence is mounting that most of the warming observed in recent decades has been caused by increasing atmospheric concentrations of greenhouse gases^{9,11,12}. Anthropogenic increases in annual-mean temperatures have also been detected on continental scales, in Europe, North America and other land regions^{13–15}. We first investigate the origins of long-term changes in decadal-mean European summer (June–August) temperatures, determining the changes attributable to anthropogenic drivers of the climate system and changes attributable to natural drivers. We then estimate how the risk of mean June–August temperatures exceeding a particular extreme threshold in any individual summer has changed as a result of this anthropogenic interference in the climate system.

Over the course of the twentieth century, June–August temperatures in Europe exhibited an overall increase, and a distinctive temporal pattern of temperature change, including cooling in the 1950s and 1960s (Fig. 1). We focus on the region bounded by 10° W and 40° E and 30–50° N (Fig. 1 inset), this being one of the regions chosen in previous studies^{13,16} to represent climatically coherent regions sufficiently large to exhibit climate change signals above the noise of natural internal variability. We use a pre-selected region in order to minimize any bias that could result from selecting our region already knowing where the most extreme temperatures occurred. Even in such a large domain, 2003 was the warmest summer on record. The history of temperature change averaged over this region is well reproduced by simulations of the HadCM3 climate model¹⁷, even at the model's relatively low spatial resolution (3.75° longitude by 2.5° latitude), when driven with both anthropogenic and natural drivers of climate change (Fig. 1; see red, green, blue and turquoise lines). Four simulations (denoted ALL) were made with different initial conditions¹⁸, each with the same combination of well mixed greenhouse gases, sulphate aerosols and changes in tropospheric and stratospheric ozone, as well as natural changes in solar output and explosive volcanic eruptions¹². A calculation of the temperature changes due to natural drivers alone (obtained by combining a simulation with solar forcing and

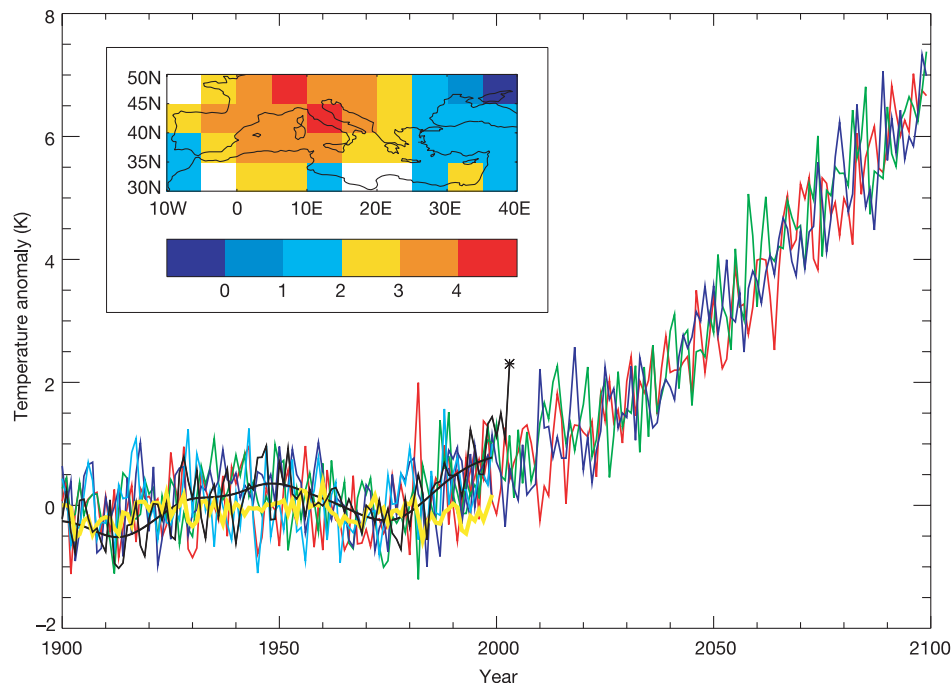


Figure 1 June–August temperature anomalies (relative to 1961–90 mean, in K) over the region shown in inset. Shown are observed temperatures (black line, with low-pass-filtered temperatures as heavy black line), modelled temperatures from four HadCM3 simulations including both anthropogenic and natural forcings to 2000 (red, green, blue and turquoise lines), and estimated HadCM3 response to purely natural forcings

(yellow line). The observed 2003 temperature is shown as a star. Also shown (red, green and blue lines) are three simulations (initialized in 1989) including changes in greenhouse gas and sulphur emissions according to the SRES A2 scenario to 2100²². The inset shows observed summer 2003 temperature anomalies, in K.

a simulation with volcanic forcing, and denoted NAT) shows no warming in the latter part of the century (Fig. 1, yellow line).

A necessary requirement for any detection of significant warming is that HadCM3 adequately represents the natural internal variability of European summer temperatures. A quantitative test, in which an estimate of the forced response calculated from the ALL ensemble mean is subtracted from the observations and the residual compared with HadCM3 simulated internal variability, shows no

significant discrepancy in variance on either interdecadal or 2- to 10-year timescales (Fig. 2). If anything, the model appears to be overestimating observed variability in this season and region, making our estimates of attributable risk relatively conservative. We also find no evidence of a secular change in variance on sub-decadal timescales in either model or observations: over the twenty-first century, the standard deviation of the forced simulations under the SRES A2 scenario (Fig. 1), when a second-order polynomial trend has been removed, increases by an insignificant 0.01 K from the first 40 years (1990–2030) to the last 40 years (2060–2100). This contrasts with the conclusions of ref. 2, although their results applied to a much smaller region.

We now apply a standard optimal detection analysis to European summer temperatures, similar to those applied to global scale patterns of temperature change^{11,12,19}. The analysis is a regression between decadal-mean seasonal-mean observations over 1920–99 and simulated temperature changes over the same period from both anthropogenic and natural forcings (ALL) and from natural forcings alone (NAT), and is in most respects identical to that of ref. 13 (see Methods). Figure 3a shows the estimated likelihood functions for the factors by which we could scale up or down the amplitudes of the model-simulated responses to anthropogenic forcing (red curve) and natural forcing (green curve) while remaining consistent with the observations. As the fifth percentile of the scaling factor on the anthropogenic response (red curve in Fig. 3a) is greater than zero, an anthropogenic influence on decadal-mean European summer temperature is detected at the 5% significance level (that is, the hypothesis that there is no positive anthropogenic influence can be rejected at the 5% level). We conclude from this investigation of decadal-mean summer temperatures that it is very likely that past anthropogenic forcing is responsible for a significant fraction of the observed European summer warming.

We now calculate the changed risk of extremely hot summers that can be attributed to past anthropogenic emissions, allowing for

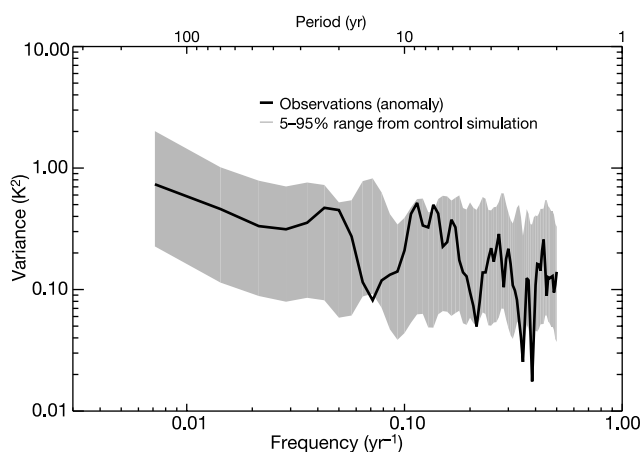


Figure 2 Power spectra of European mean summer temperature. Solid line, observed spectrum after removing an independent estimate of the externally forced response provided by the ensemble mean of the ALL simulations. Shaded region, 5 to 95 percentile region of the estimated range of spectra of natural internal variability estimated from segments taken from the HadCM3 control run of the same length as the observations. Model spectral densities have been inflated by a factor of 1.25 to allow for sampling uncertainty in the ALL ensemble mean.

uncertainty in the anthropogenic warming. Averaged over the region of interest (Fig. 1 inset), summer temperatures in 2003 exceeded the 1961–90 mean by 2.3 K (Fig. 1, black star). To quantify changes in risk, we need an objective definition of the event in question. Using 2.3 K itself is problematic for three reasons: first, relying too closely on the details of what actually occurred when defining what we are looking for introduces a selection bias in our attribution procedure; second, temperature anomalies in 2003 may have been amplified by soil-moisture feedbacks² or interactions with the North Atlantic²⁰, both of which may be under-estimated, although we do observe similar magnitude spikes in model summer temperatures in Fig. 1; third, given the length of model-simulated variability we have available, inferring the statistics of temperature excursions over 2 K requires extrapolation of extreme value distributions, which introduces further uncertainties. 2003 was the first year to reach or exceed a threshold of 1.6 K (2001 being the second-warmest European summer, at 1.5 K). We therefore consider how the probability of exceeding this threshold has changed, by comparing this estimated late-twentieth-century probability with the estimated probability of exceeding the same absolute threshold if there had been no anthropogenic influence on climate. Increasing this threshold to any value up to 2.3 K strengthens our conclusions regarding attributable risk; hence using a threshold that only just exceeds the second warmest summer is relatively conservative.

Assuming that sub-decadal continental-scale variability is stationary and adequately represented by HadCM3, we estimate possible distributions of temperatures in individual summers in the presence and absence of anthropogenic influence. We do this by adding HadCM3 control variability to reconstructions of 1990s decadal-mean temperatures both with all external factors included, and with anthropogenic factors removed, allowing for uncertainty in these

decadal-mean temperatures from the detection analysis (Fig. 3b). Figure 4a shows the estimated likelihood of the risk (probability) of exceeding a 1.6 K threshold in the presence of anthropogenic climate change (red line) and in the absence of anthropogenic change (green line), expressed both as a frequency (number of occurrences per thousand years, bottom axis) and as a return period (top axis). The clear shift from the green to the red distribution implies that an appreciable fraction of the risk of such hot summers can be attributed to human influence on climate. Even in the presence of anthropogenic warming, we conclude that the estimated probability of exceeding 1.6 K appears to be low (best estimate is a 1 in 250 year event (Fig. 4a, red curve) but this risk may be increasing rapidly).

The fraction attributable risk (FAR) is estimated in Fig. 4b (see Methods). In certain circumstances, the figure of relevance in establishing possible liability for compensation has been FAR = 0.5, corresponding to a doubling of risk over natural conditions²¹ (meaning that one event in two would have happened naturally). According to our calculation, there is a greater than 90% chance that over half the risk of European summer temperatures exceeding a threshold of 1.6 K is attributable to human influence on climate. Although there is a large spread, reflecting the remaining uncertainties in the effects of climate change on this spatial scale, the anthropogenic FAR could be substantially greater than 0.5. Also marked on Fig. 4b is a vertical line representing an overall ‘best estimate’ of the human contribution to the increased risk of these very hot European summers⁷, given the information that we have available at present. On this basis, human influence is to blame for 75% of the increased risk of such a heatwave.

Our analysis shows that European summers are warming owing to anthropogenic climate change. Under un-mitigated emissions scenarios, summers like 2003 are likely to be experienced more

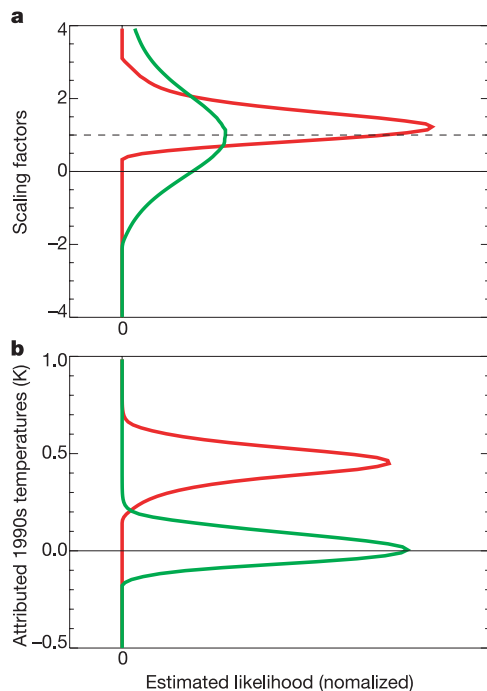


Figure 3 Estimated likelihood functions for anthropogenic and natural contributions to European summer temperature changes. The curves show estimated distributions of anthropogenic (red) and natural (green) scaling factors on model-simulated responses (**a**). 1990s summer temperatures (relative to pre-industrial climate) including all external drivers of climate change (red) and with anthropogenic drivers removed (green) (**b**). A scaling factor of zero (horizontal solid line in **a**) implies no contribution to observed 1990s temperatures from this driver, while unity (horizontal dashed line in **a**) implies no systematic under- or over-estimate by the model of the observed response to this driver. The width of these distributions reflects the uncertainties for these probabilities.

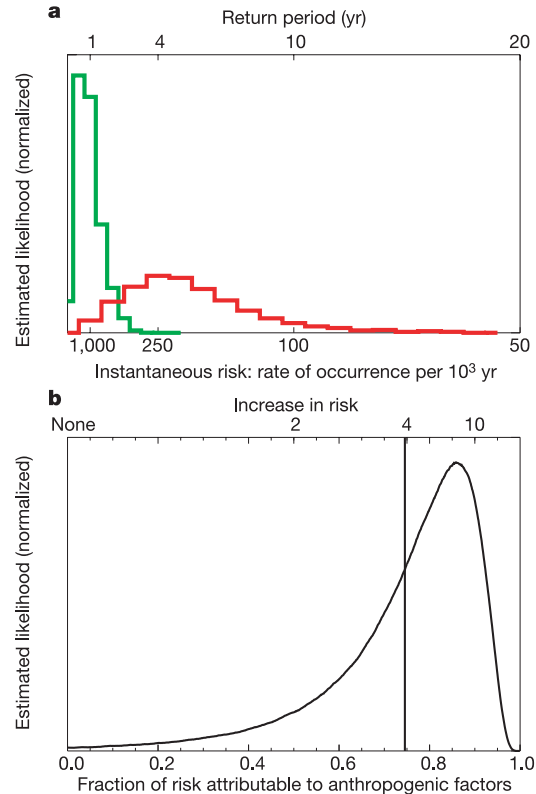


Figure 4 Change in risk of mean European summer temperatures exceeding the 1.6 K threshold. **a**, Histograms of instantaneous return periods under late-twentieth-century conditions in the absence of anthropogenic climate change (green line) and with anthropogenic climate change (red line). **b**, Fraction attributable risk (FAR). Also shown, as the vertical line, is the ‘best estimate’ FAR, the mean risk attributable to anthropogenic factors averaged over the distribution.

frequently in future; HadCM3 projections (Fig. 1) indicate that the probability of European mean summer temperatures exceeding those of 2003 increases rapidly under the SRES A2 scenario²², with more than half of years warmer than 2003 by the 2040s. By the end of this century, Fig. 1 shows that 2003 would be classed as an anomalously cold summer relative to the new climate, for the scenario and model under consideration.

We may have underestimated the FAR of a heatwave in 2003 by using 1990s figures for attributable background warming. Conversely, the FAR may have been overestimated by selecting an area that has recently been subject to extreme temperatures, although this effect should be alleviated by our use of an independently specified region. Modelling and forcing uncertainties, and any errors in the variability characteristics of the model, could mean that our assessment of the likelihood of the FAR exceeding 0.5 is in error. Including a different combination of the available natural HadCM3 simulations changes the results quoted by less than 5%, but a systematic exploration of both modelling and forcing uncertainty would require a very large multi-model ensemble, varying model parameters across their range of possible values and exploring the range of potential forcing estimates^{23,24}.

Anthropogenic warming trends in Europe imply an increased probability of very hot summers. Given the effects of the 2003 heatwave, this suggests a greater risk of associated adverse impacts, although to make a quantitative attribution assessment of any specific social, economic or ecological impact will require detailed modelling of both local meteorological conditions and their relationships with the impact in question. Nevertheless, it seems likely that past human influence has more than doubled the risk of European mean summer temperatures as hot as 2003, and with the likelihood of such events projected to increase 100-fold over the next four decades, it is difficult to avoid the conclusion that potentially dangerous anthropogenic interference in the climate system is already underway. The FAR provides a potential measure for redistributing the associated costs of such extreme events²¹. □

Methods

Attribution of decadal-mean seasonal-mean changes

In a natural extension of previous work^{12,13,25}, observed decadal-mean summer near-surface temperature changes, y , over land for 1920–99 are constructed from monthly-mean values extracted from the CRUTEM2(v) data set¹⁰ for the region 10°W–40°E, 30°–50°N, requiring data for at least two out of three months (June–August) in at least half the years. They are regressed against the mean response over the observational data-mask of a four-member ensemble of HadCM3 simulations driven with both anthropogenic and natural forcings (x_1 , ALL, red, green, blue and turquoise lines in Fig. 1) and a second set of simulations of the response to solar and volcanic forcing alone (x_2 , NAT, yellow line in Fig. 1) plus noise:

$$y = (x_1 - \nu_1)\beta_1 + (x_2 - \nu_2)\beta_2 + \nu_0 \quad (1)$$

where β_1 , β_2 are the unknown scaling factors to be estimated in the regression, ν_0 is the noise in the observations, and ν_1 and ν_2 account for sampling uncertainty introduced by estimating model-simulated responses from a finite ensemble²⁶. A scaling factor of 1 would imply that the response in the model simulations is identical to the observed changes, while a factor of 0 implies no correspondence between modelled and observed responses. Likelihood functions for scaling factors obtained from the regression account only for sampling variability that arises from natural internal climate variability as represented by HadCM3. They do not account for systematic errors in the shapes of the modelled patterns or uncertainties due to missing forcings (for example, land use changes or fossil-fuel black carbon emissions). If we assume a uniform prior in these scaling factors, these likelihoods can be thought of as probability distributions.

The combined effects of solar and volcanic forcings are estimated from a simulation with volcanic forcing amplified by a factor of 10 and solar forcing amplified by a factor of 5, multiplied by 0.1 and 0.2, respectively, and added. This approach was taken in order to obtain the clearest possible climate signal from a weak forcing and a limited number of climate simulations. Previous work showed that the large-scale temperature responds linearly to increasing these forcings²⁷. Including an additional four simulations with unamplified natural forcings in the analysis makes little difference to the results.

We use the HadCM3 control run and intra-ensemble variability to estimate internal variability, ν_0 , ν_1 and ν_2 , taking into account the enhanced signal-to-noise from averaging over the ALL ensemble and inflating the solar and volcanic forcing¹³. This gives scaling factors for the combined effects of all forcings and for natural forcings, from which the anthropogenic and natural scaling factors can be calculated by linear transformation²⁷ (Fig. 3a). Where the 5 to 95 percentile uncertainty range does not include zero scaling factor, this indicates that the relevant signal has been detected at the 5% significance level,

that is, there is less than 5% chance that the relevant forcing has had no effect. From the scaling factors and their uncertainties, we derive likelihood functions of 1990s temperature anomalies attributable to the combined effects of all forcings and attributable to natural factors alone (Fig. 3b).

Attribution of change in risk of exceeding a threshold

The distribution of attributed anthropogenic decadal-mean warming in the 1990s is used to calculate the FAR for an individual European summer exceeding the 1.6 K threshold as follows. We estimate the probability of exceeding the 1.6 K threshold from the generalized Pareto distribution (GPD) fitted to the HadCM3 control run temperatures²⁸. The spectral analysis shown in Fig. 2 indicates that HadCM3 provides an adequate representation of internal variability. We calculate the probability, P_0 , of the 1.6 K threshold being exceeded without anthropogenic climate change (shown by the green curve in Fig. 4a, expressed as return period, top axis, and instantaneous risk: rate of occurrence per 10^3 yr, bottom axis) and the probability, P_1 , of exceeding the threshold with anthropogenic climate change (red curve in Fig. 4a). In estimating the green curve, the chance of exceeding the 1.6 K threshold is re-estimated using the GPD with the mean summer temperature adjusted to each percentile of the estimated distribution of decadal-mean temperature anomalies attributable to natural forcing and internal variability. For the red curve, the response to anthropogenic forcing is also included. A bootstrap resampling method is used to allow for uncertainty in GPD parameters. From these distributions, we calculate the distribution of attributable increase in risk (P_1/P_0) and the FAR ($(P_1 - P_0)/P_1$), as shown in Fig. 4b, whose spread reflects uncertainty in the magnitude of the forced decadal-mean response and in the estimation of the extreme value distribution parameters due to limited sample size.

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Evidence for cultivar adoption and emerging complexity during the mid-Holocene in the La Plata basin

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Multidisciplinary investigations at the Los Ajos archaeological mound complex in the wetlands of southeastern Uruguay challenge the traditional view that the La Plata basin was inhabited by simple groups of hunters and gatherers for much of the pre-Hispanic era^{1–4}. Here we report new archaeological, palaeo-ecological and botanical data indicating that during an increasingly drier mid-Holocene, at around 4,190 radiocarbon (¹⁴C) years before present (BP), Los Ajos became a permanent circular plaza village, and its inhabitants adopted the earliest cultivars known in southern South America. The architectural plan of Los Ajos during the following Ceramic Mound Period (around 3,000–500 ¹⁴C yr BP) is similar to, but earlier than, settlement patterns demonstrated in Amazonia^{5–10}, revealing a new and independent architectural tradition for South America.

Research on the emergence of complex societies in South America has mainly concentrated on Andean coastal and highland valleys^{11–14}, and more recently in the lowland forest and riverine regions of Amazonia^{5–10}. The La Plata basin (Fig. 1a) is a large and little explored river system that is beginning to reveal an early and long sequence of unique and complex cultural trajectories. The natural environment of the study area is dominated by subtropical grasslands interspersed with vast extensions of wetlands. In strategic locations circumscribed to wetland floodplains, archaeological mound complexes are large and numerous. They have mounded architecture geometrically arranged in circular, elliptical and horseshoe formats that surround a central communal space (Fig. 1b).

The first excavations at the multi-mound site called Los Ajos, by Bracco¹⁵, in the early 1990s consisted of a block excavation in Mound Alfa, a test unit in Mound Beta and a few opportunistic test units in off-mound areas. This work established the mid-Holocene age of the earthen mounds in the area. The Pre-ceramic Mound Period (PMP) component at Los Ajos yielded five dates between 3,950 and 3,350 ¹⁴C yr BP (4,580 and 3,380 calibrated (cal.) yr BP)¹⁶. Closely comparable dates from the deeper PMP components of the Puntas de San Luis, Isla Larga and Potrerillo sites

collectively ascertained the antiquity of the PMP^{17–19} (Table 1).

Our new excavation programme consisted of the placement of a block excavation in Mound Gamma, a test unit in Mound Delta, two trench transects articulating mound and off-mound areas and a 50-m systematic interval transect sampling strategy of test units to target off-mound areas (Fig. 2) totalling an excavated area of 305 m² (ref. 20). Our work revealed that Los Ajos is one of the largest and most formally laid out sites in the study area and covers about 12 ha (Fig. 2a). Its Inner Precinct includes six flat-topped, quadrangular platform mounds (called 6, Alfa, Delta, Gamma, 4 and 7) closely arranged in a horseshoe formation and with a height above ground level of 1.75 to 2.5 m (Fig. 2a, b). Two dome-shaped mounds (called Beta and 8) frame the central, oval plaza with a size of 75 × 50 m (Supplementary Fig. 1a). The formal and compact Inner Precinct contrasts with more dispersed and informally arranged peripheral sectors, which include two crescent-shaped rises (named TBN (Supplementary Fig. 1b) and TBS), five circular and three elongated lower dome-shaped mounds, borrow pits and a vast off-mound area bearing subsurface occupational refuse.

A series of major social and economic changes took place at Los Ajos during the PMP. The broad contemporaneity of radiocarbon dates (Table 1), artefact content and similarities in Pre-ceramic Mound Component (PMC) stratigraphy among mounds Alfa, Delta and Gamma suggest that the Los Ajos inhabitants began to live in a circular household-based community, partitioning the site into a number of discrete functional areas characterized by the placement of residential units around a central plaza area. Charcoal from the basal level of Mound Gamma, 270–275 cm deep (arbitrary depth), dates the beginning of the PMC at 4,190 ± 40 ¹⁴C yr BP (4,840–4,580 cal. yr BP). Another radiocarbon assay at 205–210 cm deep yielded a date of around 3,460 ¹⁴C yr BP (3,980–3,470 cal. yr BP). The upper portion of the PMC in Mound Delta yielded a date of around 2,960 ± 120 ¹⁴C yr BP. Taken together, the eight dates from Los Ajos place the PMC occupation between 4,190 ± 40 and 2,960 ± 40 ¹⁴C yr BP. The two oldest dates from the basal levels of the PMC at Mound Gamma and Alfa suggest that mound-building began between around 4,190 and 3,950 ¹⁴C yr BP (4,840–4,160 cal. yr BP). Mounds grew as a result of multiple overlapping of domestic occupations where a wide range of activities associated with food preparation, consumption, stone tool production and maintenance took place. The PMC Layer 4 is characterized by a ~85-cm-thick compact, very dark brown silty loam organic sediment (Fig. 3) consisting of relatively undifferentiated deposits composed of lithic debitage and tools, small fragments of charred bone, ash and soot lenses, and small pieces of burned clay.

Our associated palaeoecological data²⁰ indicate that, similar to other regions in the Americas²¹, the mid-Holocene (between 6,620 ± 40 ¹⁴C yr BP (7,580–7,440 cal. yr BP) and 4,020 ± 40 ¹⁴C yr BP (4,570–4,410 cal. yr BP)) was a period of significant environmental flux marked by increasing aridity. At around 4,020 ¹⁴C yr BP (4,570–4,410 cal. yr BP) a maximum drying episode occurred, as evidenced by a massive spike of *Amaranthaceae*/*Chenopodiaceae* coupled with a sharp drop in wetland species (Supplementary Fig. 2). These changes probably caused a decrease in the surface water recharge to the inland wetlands and waterways. Although reduced in extent, wetlands probably became attractive places for pre-Hispanic populations by providing abundant, now more highly circumscribed plant and animal resources and a stable source of water. The mid-Holocene drying trend may thus have acted as an important catalyst for the reorientation of settlements towards the topographically higher freshwater wetlands, where permanent communities were established.

Despite the application of an intensive flotation program, seeds, roots and nuts were not recovered; however, phytoliths and starch grains were abundant (Methods are available as Supplementary Information). Phytoliths diagnostic of maize cobs²² (Fig. 4c) record their earliest appearance in the level from 255–260 cm deep, 15 cm

above the earliest dated context of the PMC (about 4,190 ^{14}C yr BP), and continue to be present throughout the sequence (Table 1). Starch grains diagnostic of maize kernels²³ (Fig. 4d) were recovered from a subspherical mano excavated at a depth of 205–210 cm, 5 cm above a date of about 3,460 ^{14}C yr BP (3,980–3,470 cal. yr BP) (Supplementary Fig. 3a). Large spherical scalloped phytoliths diagnostic of fruit rinds from domesticated species of *Cucurbita*²⁴ (Fig. 4e) occurred for the first time in PMC contexts at a depth of 255 cm, 15 cm above the earliest dated context around 4,190 ^{14}C yr BP, and continue to be present throughout the sequence (Supplementary Table 1). Palm phytoliths were also present throughout

the sequence. In addition, starch grains from maize kernels were documented in contexts dating to 3,600 ^{14}C yr BP at Isla Larga and 2,800 ^{14}C yr BP at Los Indios, two other mound sites in the study area. At Isla Larga, starch grains from *Phaseolus* sp. beans (Fig. 4a) were derived from contexts dated to 3,050 ^{14}C yr BP, and starch grains from *Canna* sp. rhizomes (Fig. 4b) were evidenced from contexts dated to 3,660 ^{14}C yr BP²⁵. Larger preceramic mound complexes are situated along fertile wetland floodplains, suggesting that PMP people engaged in wetland margin horticulture during late spring and early fall when the water table is at its lowest and the organic, fertile and easy-to-till superficial peat horizon is readily exposed.

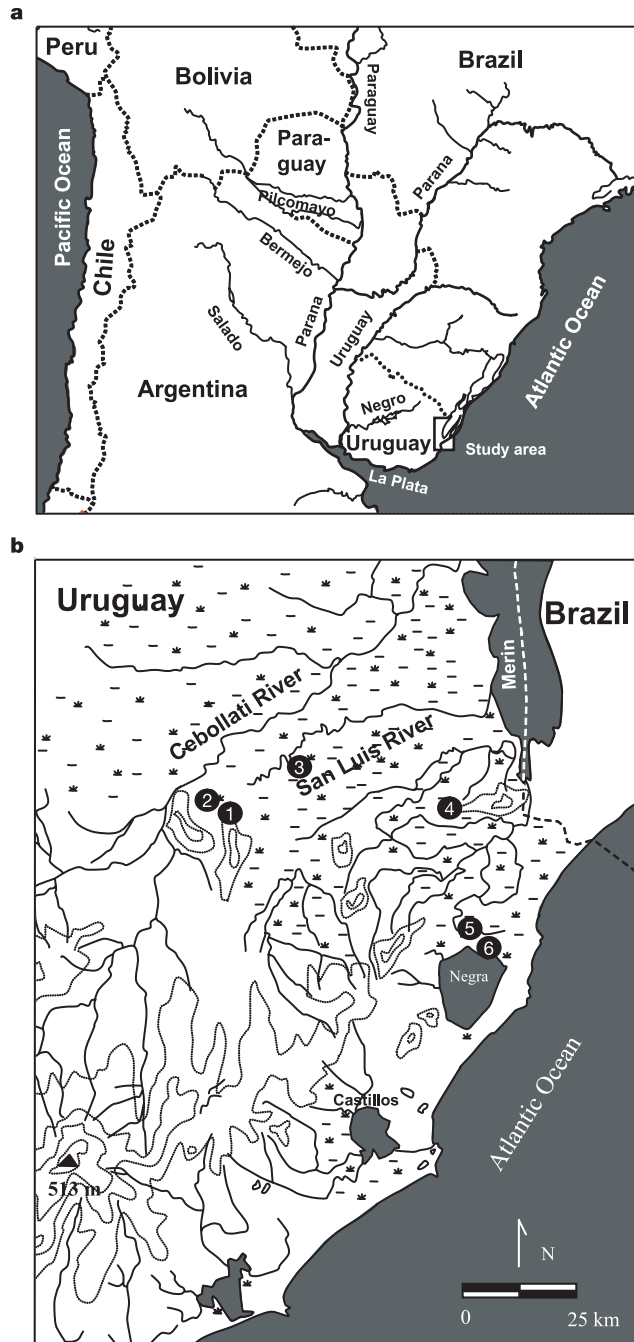


Figure 1 Maps showing the position of the study area and locations of archaeological sites within the study area. **a**, Location of the study area and the La Plata basin. **b**, Map of southeastern Uruguay showing archaeological sites. Key: 1, Los Ajos; 2, Estancia Mal Abrigo; 3, Puntas de San Luis; 4, Isla Larga; 5, Los Indios; 6, Potrerillo.

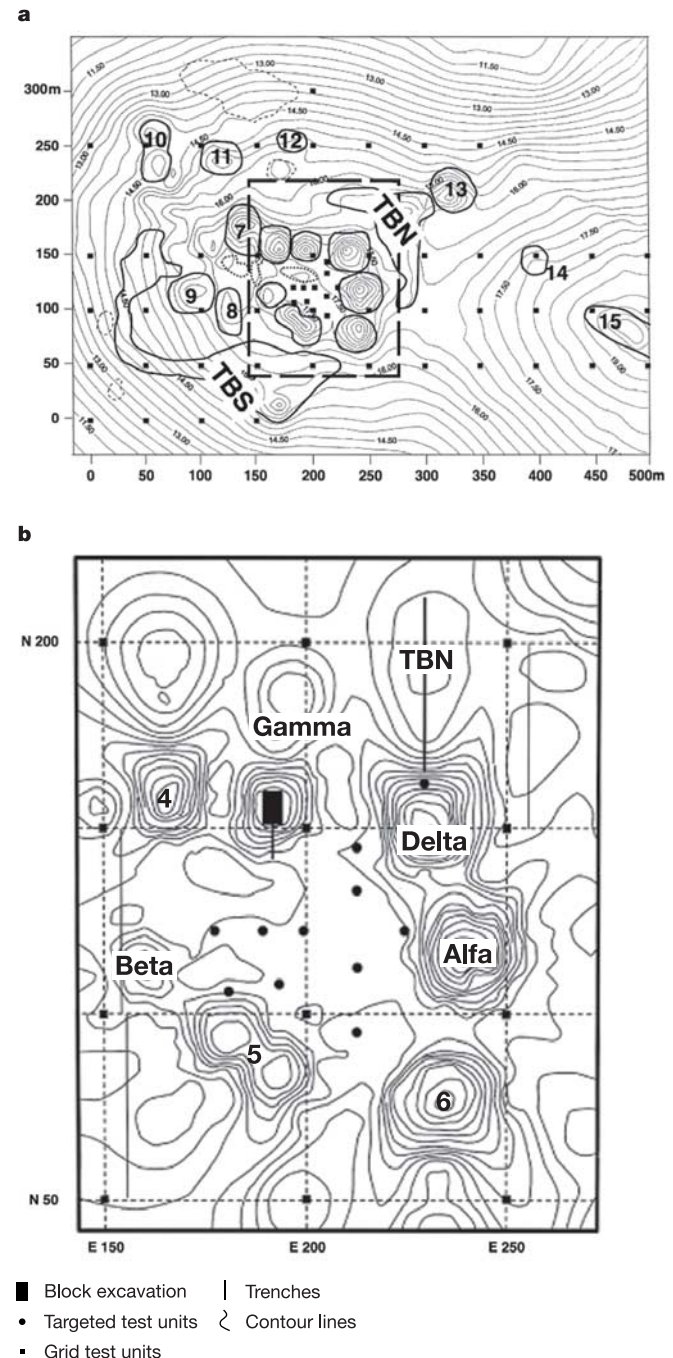


Figure 2 Topographical maps of the study site. **a**, Topographical and planimetric map of Los Ajos. Countour intervals, 0.33 m. Thick lines show perimeter of mounds. **b**, Topographical map of the Inner Precinct of Los Ajos. Countour intervals, 0.2 m. (Modified from Bracco¹⁹).

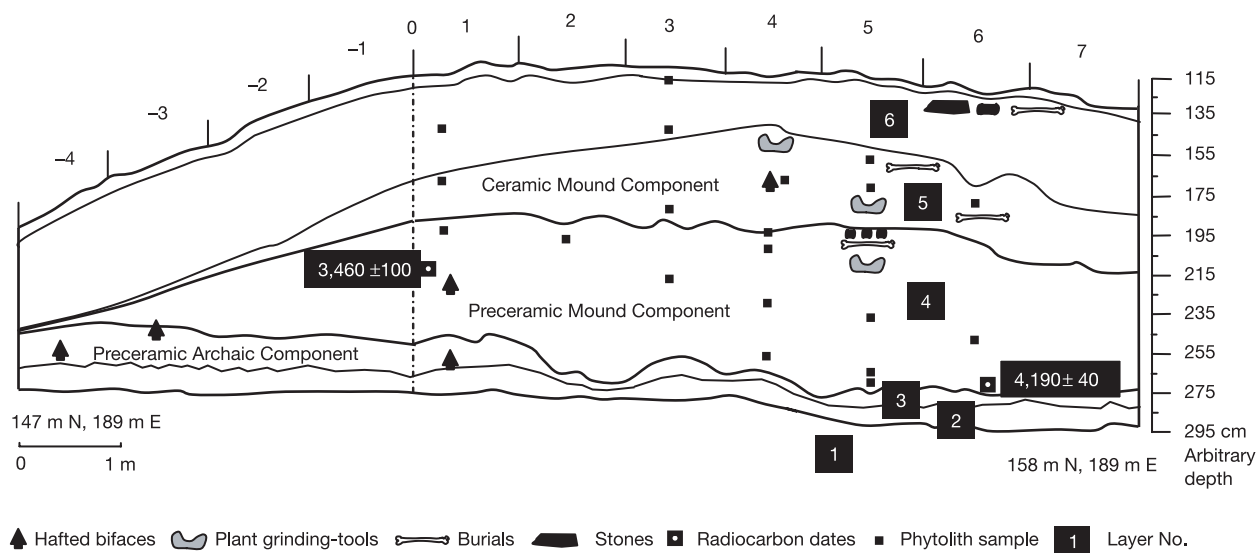


Figure 3 West wall stratigraphic sketch of Mound Gamma block excavation and part of trench. Vertical exaggeration $\times 2$.

During the following Ceramic Mound Period (CMP) occupation dated between $\sim 3,000$ and 500 ^{14}C yr BP¹⁷, Los Ajos experienced the formalization and spatial segregation of its mounded architecture. Mound Gamma's Layers 5 and 6 consist of a dark brown sediment bearing a medium to high concentration of gravel within a mottled silt loam matrix (Fig. 3) containing burned clay, charcoal and ash lenses. Capping episodes consisting of gravel loads remodelled Mound Gamma from the extant PMP $0.6\text{--}0.8$ m high, circular, dome-shaped mound to a larger, quadrangular, 1.40 m tall, flat-topped, bevelled-edged platform mound. The presence of similar gravelly layers in Mound Alfa and Delta attest that the remodelling of mounds was a generalized practice at Los Ajos. The horseshoe arrangement of imposing platform mounds, the TBN and Mound 13 appear to represent an integrated architectural plan oriented to the north-east that contrasts with the less conspicuous and informally arranged southwestern sector, marking an asymmetrical distribution of architecture in the Inner Precinct.

Around $1,660$ ^{14}C yr BP, the TBN and the TBS experienced substantial accumulation of occupational refuse attaining up to 0.80 m of anthropic deposits, and indicating a more intense and

permanent occupation of the site. Subsurface occupational refuse distributed over the vast peripheral off-mound areas covering over 12 ha suggest a large resident population for the site during the CMC. There is no evidence of major changes in the Los Ajos food economy during this period. Maize cob phytoliths and maize starch grains were recovered from two milling stone bases^{22,23} (Supplementary Fig. 3b, c) and continue to appear in the archaeological sediments (Supplementary Table 1) along with domesticated squash phytoliths. Maize leaf cross-shaped phytolith assemblages were detected^{26–28} in the central part of the TBN trench transect, showing that maize was planted and/or husked in this part of the site (Supplementary Table 2; Fig. 4f). The ceramic assemblage recovered at Los Ajos closely resembles the broadly defined Vieira tradition² dated to around $2,500$ ^{14}C yr BP, which is widely distributed over southeastern Uruguay and the Brazilian state of Rio Grande do Sul.

The unexpected cultural sequence at Los Ajos reveals an early expression of social complexity not registered before now in this region of southern South America. Our data correspond with cultural developments recently documented during the mid- and late Holocene in Amazonia^{5–10}, the Atlantic coast of Brazil²⁹ and

Table 1 Preceramic Mound Component radiocarbon dates from southeastern Uruguay

Provenience (site)	Arbitrary depth (cm)	Laboratory number	Dated material	Conventional ^{14}C yr BP	2-Sigma cal. yr BP
Los Ajos					
TBN Trench					
Sector 7	160–165	Beta-158278	Charcoal (AMS)	$1,050 \pm 40$	$1,050\text{--}920$
Sector 6	190–195	Beta-158281	Charcoal (AMS)	$1,660 \pm 40$	$1,690\text{--}1,660$
Mound Delta	205–210	Beta-158277	Charcoal	$2,960 \pm 120$	$3,400\text{--}2,740$
Mound Gamma					
Sector 1/D	210–215	Beta-158279	Charcoal	$3,460 \pm 100$	$3,980\text{--}3,470$
Sector 6/C	270–275	Beta-158280	Charcoal (AMS)	$4,190 \pm 40$	$4,840\text{--}4,580$
Mound Alfa					
Layer III	280–285	URU 0052	Charcoal	$3,350 \pm 90$	$3,830\text{--}3,380^*$
	285–290	URU 0033	Charcoal	$3,870 \pm 280$	$5,030\text{--}5,010$ and $4,990\text{--}3,550^*$
	295–300	URU 0034	Charcoal	$3,690 \pm 270$	$4,830\text{--}3,370^*$
	340–345	URU 0089	Charcoal	$3,950 \pm 80$	$4,580\text{--}4,160^*$
	345–355	URU 0088	Charcoal	$3,750 \pm 140$	$4,520\text{--}4,470$ and $4,450\text{--}3,710^*$
Puntas de San Luis					
Mound II	Layer II	URU 009	Charcoal	$3,550 \pm 60$	$3,980\text{--}3,680^*$
	Layer III	URU 009	Charcoal	$3,650 \pm 50$	$4,100\text{--}3,840^*$
	Layer III	URU 010	Charcoal	$3,730 \pm 100$	$4,410\text{--}3,830^*$
Isla Larga					
Mound I	260–270	URU013	Charcoal	$3,660 \pm 120$	$4,380\text{--}3,670^*$
		URU014	Charcoal	$3,630 \pm 60$	$4,100\text{--}3,820^*$
Potreriillo					
Mound I	Basal level	URU 083	Charcoal	$3,790 \pm 90$	$4,420\text{--}3,900^*$
		URU 165	Charcoal	$3,820 \pm 100$	$4,510\text{--}4,480$ and $4,440\text{--}3,910^*$

* $^{13}\text{C}/^{12}\text{C}$ ratio estimated.

possibly the Pantanal³⁰, regions that along with the La Plata basin had previously been considered marginal^{1–4} when compared with the states and chiefdoms of the Andes and Mesoamerica. Our data also provide the first evidence of permanent village living in southeastern South America by people who subsisted on mixed economies and adopted major crop plants such as maize (*Zea mays* L.) and squash (*Cucurbita* spp.) long before previously thought^{1–4}. The palaeoecological data show that, as in other regions of the world²¹, the mid-Holocene was characterized by significant climatic and ecological changes, and that these perturbations were associated with important cultural transitions involving permanent mounded settlements situated within resource rich, circumscribed wetlands. The presence of at least four excavated mound complexes (Los Ajos, Puntas de San Luis, Isla Larga and Potrerillo (Fig. 1b)) with broadly contemporaneous PMP dates, suggests that southeastern Uruguay was a locus of early population concentration in lowland South America. The later CMP formal architectural plan of Los Ajos is earlier and sufficiently different from similar developments in Amazonia^{5–10} as to indicate an independent architectural tradition for the region. The results of this study will now allow for a broader consideration of the part that dynamic human–environment

interactions, imported cultivars and social conditions played in the emergence of early complex societies in the La Plata basin. □

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Author contributions J.I. directed the multidisciplinary research and conducted the archaeological, lithic and phytolith analyses. I.H. undertook the starch grain analysis, C.L. did the pollen analysis, O.M. directed the lithic debitage and ceramic analysis, A.R. carried out the faunal analysis and E.A. collected and identified the selected plant species to build the phytolith reference collection of southeastern Uruguay.

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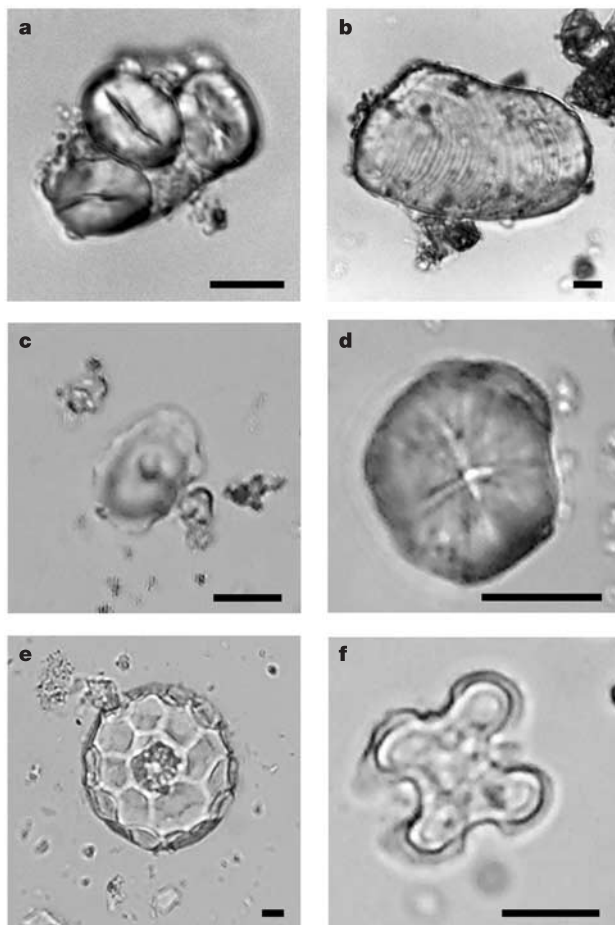


Figure 4 Selected phytoliths and starch grains. **a**, *Phaseolous* sp. starch grains (Los Indios, Mound III, 120–130 cm). **b**, *Canna* sp. starch grain (Isla Larga, Mound I, 275 cm deep). **c**, *Zea* sp. specific ruffle-top rondel phytolith (Los Ajos, Mound Gamma, 155–160 cm deep, sector 3/E). **d**, *Zea mays* starch grain (Los Ajos, Mound Gamma, 155–160 cm deep, sector 3/E). **e**, *Cucurbita* sp. spherical scalloped phytolith (Los Ajos, Mound Gamma 180–185 cm deep, sector 3/B). **f**, *Zea mays* large Variant 1 cross-shaped phytolith (Los Ajos, TBN, 170–175 cm deep, sector 7). Scale bar, 10 μ m.

***Trichomonas* hydrogenosomes contain the NADH dehydrogenase module of mitochondrial complex I**

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Hydrogenosomes are double-membraned ATP-producing and hydrogen-producing organelles of diverse anaerobic eukaryotes¹. In some versions of endosymbiotic theory they are suggested to be homologues of mitochondria^{2–4}, but alternative views suggest they arose from an anaerobic bacterium that was distinct from the mitochondrial endosymbiont^{5,6}. Here we show that the 51-kDa and 24-kDa subunits of the NADH dehydrogenase module in complex I, the first step in the mitochondrial respiratory chain⁷, are active in hydrogenosomes of *Trichomonas vaginalis*. Like mitochondrial NADH dehydrogenase, the purified *Trichomonas* enzyme can reduce a variety of electron carriers including ubiquinone, but unlike the mitochondrial enzyme it can also reduce ferredoxin, the electron carrier used¹ for hydrogen production. The presence of NADH dehydrogenase solves the long-standing conundrum of how hydrogenosomes regenerate NAD⁺ after malate oxidation. Phylogenetic analyses show that the *Trichomonas* 51-kDa homologue shares common ancestry with the mitochondrial enzyme. Recruitment of complex I subunits into a H₂-producing pathway provides evidence that mitochondria and hydrogenosomes are aerobic and anaerobic homologues of the same endosymbiotically derived organelle.

The evolutionary origins of *Trichomonas* hydrogenosomes remain debated because, unlike mitochondria or plastids, they lack an associated genome⁸. Because the phylogenetic position of *Trichomonas* is unresolved, its relationship to mitochondrion-bearing eukaryotic lineages is also unclear⁴. Characters suggesting that *Trichomonas* hydrogenosomes and mitochondria share a common origin include the surrounding double membrane, a common mode of division, similar overall physiology, common protein import pathways and conserved mechanisms of iron–sulphur-cluster assembly^{9–12}. However, *Trichomonas* hydrogenosomes also contain enzymes that are typically found in anaerobic bacteria¹, including pyruvate:ferredoxin oxidoreductase and the iron [Fe]-hydrogenase that makes hydrogen. The presence of these enzymes, which are atypical for mitochondria, has recently⁶ been used to resurrect an old hypothesis⁵ for the origins of hydrogenosomes through symbioses involving anaerobic bacteria distinct from the mitochondrial endosymbiont.

We identified homologues in *Trichomonas vaginalis* of the mitochondrial 51-kDa (NuoF, named Tvh-47) and 24-kDa (NuoE, named Tvh-22) subunits of the catalytic flavoprotein component of mitochondrial complex I (Fig. 3). Complex I, also called NADH:ubiquinone oxidoreductase, is the first part of the mitochondrial respiratory chain⁷, so its presence in *Trichomonas*—which lacks cytochromes¹—was unexpected. Conceptual translation of both genes revealed that they preserve key functional residues⁷ (Fig. 3) and both contain putative leader sequences resembling those on proteins known to localize to the *Trichomonas* hydrogenosome¹³. Localization experiments on cell fractions with the use of a specific antibody raised against the recombinant *Trichomonas*

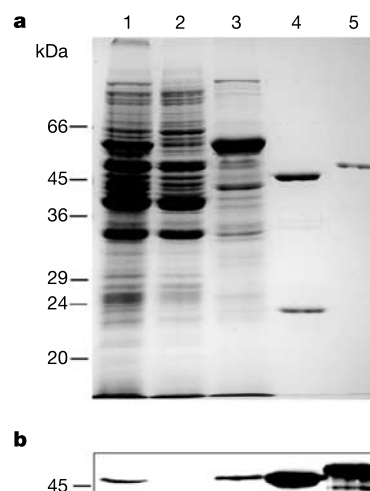


Figure 1 Localization of *Trichomonas* NADH dehydrogenase subunits. **a**, Coomassie-stained SDS–polyacrylamide-gel electrophoresis analysis of *Trichomonas* subcellular fractions: total homogenate (lane 1), cytosol (lane 2) and hydrogenosomes (lane 3). Lane 4, subunits of NADH dehydrogenase (Tvh-47 and Tvh-22 proteins (apparent molecular mass 47 and 23 kDa, respectively)) purified to homogeneity from *T. vaginalis* hydrogenosomes. Lane 5, recombinant Tvh-47 subunit (apparent molecular mass 54 kDa). **b**, Parallel analysis of the proteins by western blotting and the anti-Tvh-47 polyclonal antibody.

51-kDa homologue revealed that the protein is localized to the hydrogenosomal fraction (Fig. 1); this is confirmed by homologous transfection experiments demonstrating that Tvh-47 co-localizes with malic enzyme, a biochemical marker¹⁴ for the *Trichomonas* hydrogenosome (Fig. 2). Mass spectrometry of the active NADH dehydrogenase purified from *Trichomonas* hydrogenosomes confirmed the identity of the Tvh-47 and Tvh-22 proteins (Figs 1 and 3).

Uncovering the evolutionary origins of these *Trichomonas* pro-

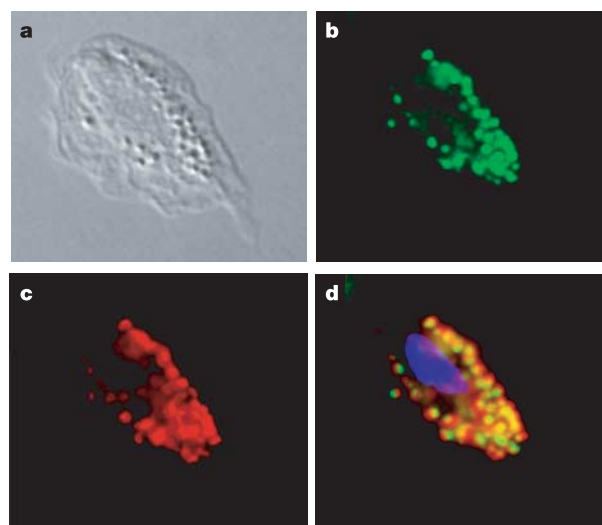


Figure 2 Localization of Tvh-47 within *Trichomonas* hydrogenosomes. **a**, Differential interference contrast image of a single transfected *Trichomonas vaginalis* cell. **b**, The same cell expressing an episomal plasmid containing Tvh-47 tagged with a carboxy-terminal haemagglutinin tag, probed with a mouse anti-haemagglutinin monoclonal antibody and Alexa Fluor-488 (green) donkey anti-mouse Ig. **c**, Localization of the hydrogenosomal marker protein malic enzyme, using rabbit anti-(malic enzyme) polyclonal antibody and Alexa Fluor-546 (red) donkey anti-rabbit Ig. **d**, Merge of **b** and **c** showing co-localization of the two proteins. The nucleus was stained blue with 4',6-diamidino-2-phenylindole.

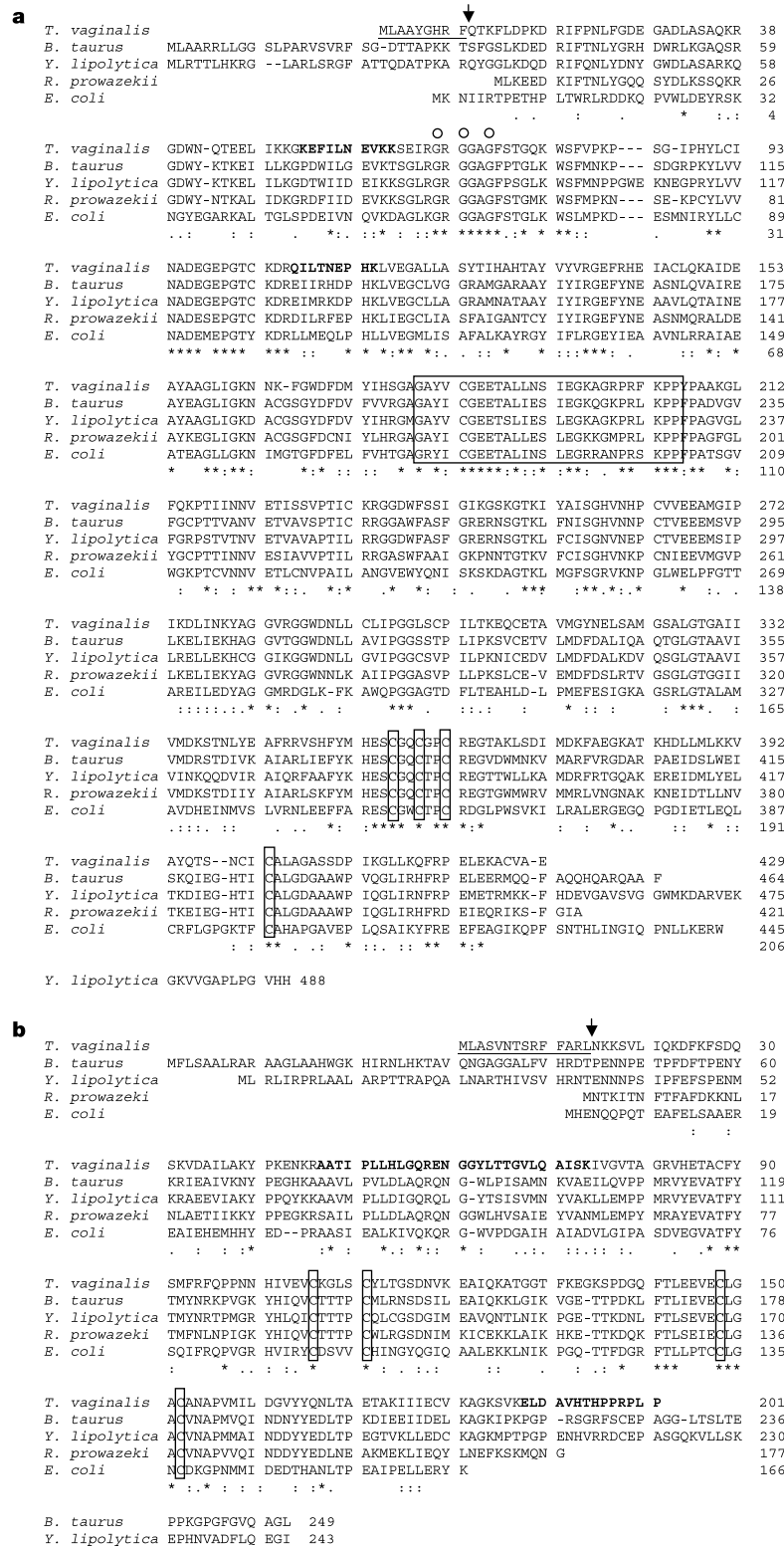


Figure 3 Sequences of NuoF and NuoE. **a**, Sequence alignment of *Trichomonas vaginalis* 47-kDa subunit of complex I (NuoF) with mitochondrial (*Bos taurus*, GenBank A39362; *Yarrowia lipolytica*, AAF65194) and eubacterial (*Rickettsia prowazekii*, NP_22050; *Escherichia coli*, NP_311195) homologues. Glycines of the NADH-binding motif GXGGXGX₃G are indicated by open circles. The FMN-binding site (GA/MGA/RVY/ICGEERA/SL/IE/NSL/IEG) and conserved cysteines motif coordinating the [4Fe–4S] cluster (CX₂CX₂CX_{38–40}C) are boxed. **b**, Sequence alignment of *T. vaginalis* 22-kDa subunit of complex I (NuoE) with mitochondrial (*Bos taurus*, B30113; *Yarrowia lipolytica*,

CAB65523) and eubacterial (*Rickettsia prowazekii*, NP_220737; *Escherichia coli*, NP_311196) homologues. Invariable cysteines coordinating the [2Fe–2S] cluster (CX₄CX_{35–36}CX₃C) are boxed. N-terminal pre-sequences of *T. vaginalis* subunits are underlined. An arrow indicates cleavage sites predicted by PSORT II (<http://psort.nibb.c.jp/>). Sequences obtained by matrix-assisted laser desorption/ionization Q-TOF are in bold. Asterisks indicate fully conserved residues, colons strongly conserved residues, and full stops weakly conserved residues.

teins pushes phylogenetics to its limits because, like many parasite proteins, the *Trichomonas* proteins are divergent (Fig. 3). TvH-22 was too poorly conserved and short to yield much insight into its origins, so we concentrated on the longer TvH-47. Initial phylogenetic analyses of TvH-47 and 51-kDa proteins, using a gamma-correction for site-rate variation and the empirical WAG matrix¹⁵, placed the *Trichomonas* sequence outside the mitochondrial and alpha-proteobacterial sequences. However, we also recovered alternative trees that placed the *Trichomonas* TvH-47 protein within the mitochondrial clade that could not be rejected with the AU test¹⁶. Notably, the *Trichomonas* branch was much longer than the other branches in the tree and the TvH-47 protein had a different amino acid composition from the other sequences, phenomena that are known to cause severe problems for phylogenetic analyses^{17,18}. To mitigate these phenomena we recoded each amino acid according to the six groups of chemically related amino acids that commonly replace one another^{15,19,20} and reanalysed the data. This recoding is related to transversion analysis and RY coding²¹ of DNA sequences, and like these methods had the effect of shortening long branches and homogenizing the amino acid composition between sequences. It also allowed us to estimate a general time-reversible

matrix specific to our data set. In these analyses, the best tree placed the *Trichomonas* TvH-47 sequence within the mitochondrial clade (Fig. 4, Supplementary Table 1 and Supplementary Fig. 1). Thus, at the very least, our analyses cannot reject the hypothesis of a common origin for the *Trichomonas* and mitochondrial 51-kDa proteins, and the more sophisticated analysis prefers it.

Mitochondrial complex I catalyses the transfer of electrons from NADH to the lipid-soluble electron carrier ubiquinone in the respiratory chain⁷. The NADH dehydrogenase activity is associated with the 51-kDa and 24-kDa subunits of complex I. *Trichomonas* TvH-47 and TvH-22, which co-purify as an active heterodimeric enzyme from hydrogenosomes (Fig. 1, Supplementary Table 2), reduced, in an NADH-dependent manner, several electron carriers (Supplementary Table 3) including ubiquinone and *Trichomonas* 2Fe-2S ferredoxin. Kinetic data suggest that ferredoxin is the natural electron acceptor in the hydrogenosome (Supplementary Table 4). The *Trichomonas* enzyme is insensitive to rotenone, which blocks electron transfer in mitochondria by binding to complex I subunits that form the ubiquinone binding site; such units are missing from the *Trichomonas* enzyme.

The presence in *Trichomonas* hydrogenosomes of the NADH

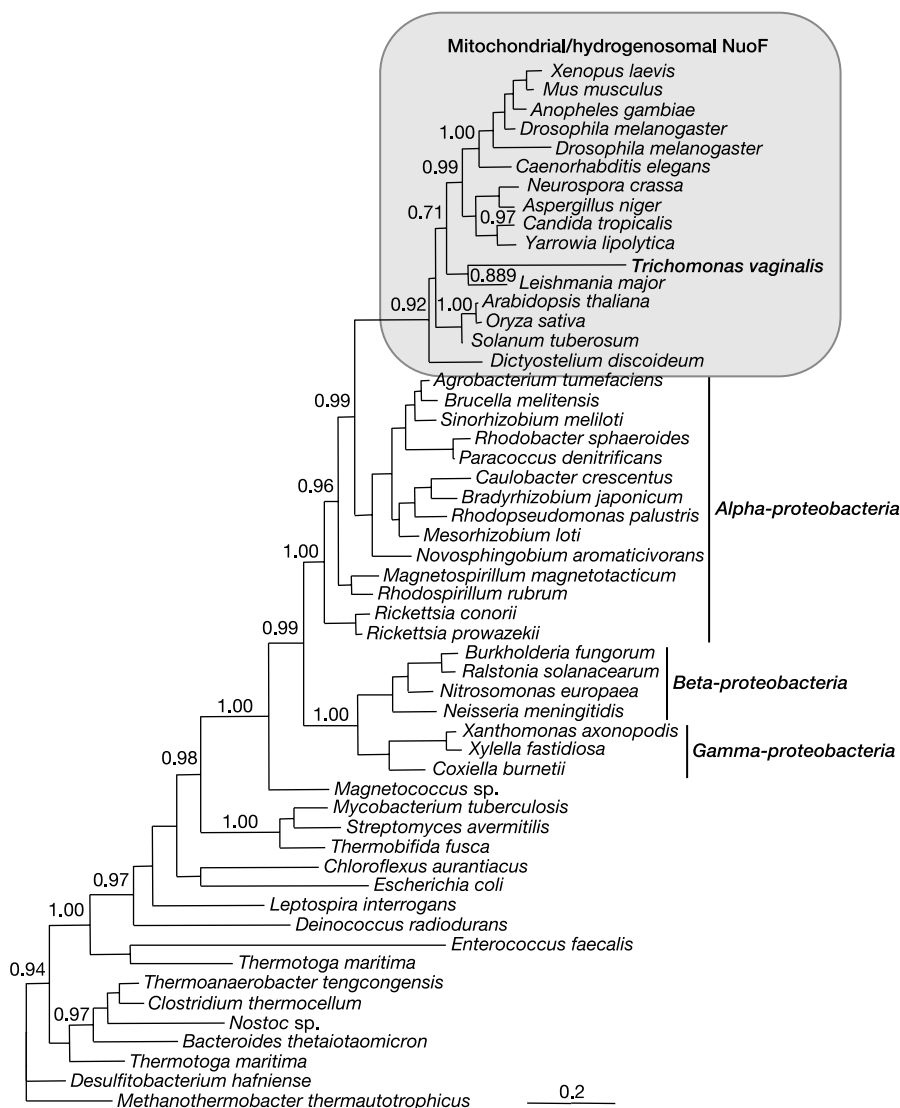


Figure 4 Phylogenetic analyses of NuoF homologues. Bayesian consensus tree of a general time-reversible substitution matrix estimated¹⁸ from amino acid sequences recoded into the six Dayhoff groups: ASTGP, DNEQ, RKH, MVIL, FYW and C¹⁹. Among-site rate heterogeneity was modelled by using a four-category gamma correction with a

fraction of invariant sites (pinvar). All parameters, including the composition and substitution rate matrix, were free, and the analysis used the Metropolis-coupled MCMC strategy from MrBayes²⁹. Posterior probabilities for selected branches are shown at nodes. Scale bar indicates number of changes per site.

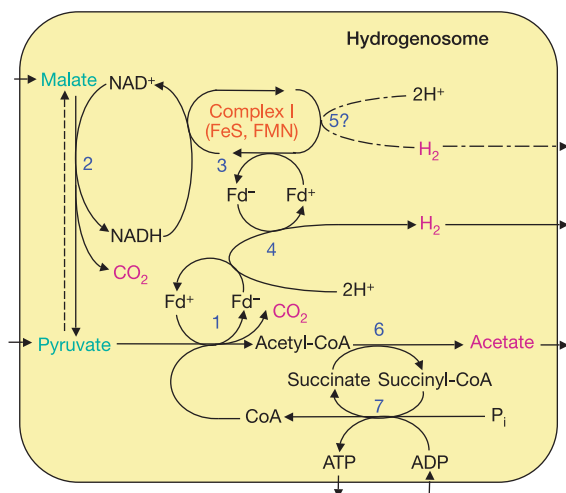


Figure 5 A schematic diagram illustrating the possible roles of NADH dehydrogenase from complex I in hydrogenosomal metabolism of *Trichomonas vaginalis*. Enzymes are indicated as follows: 1, pyruvate:ferredoxin oxidoreductase; 2, NAD-dependent malate dehydrogenase (decarboxylating); 3, NADH:ferredoxin oxidoreductase activity of the 51-kDa (Tvh-47) and 24-kDa (Tvh-22) catalytic flavoprotein component of complex I; 4, ferredoxin-dependent Fe-hydrogenase; 5, hypothetical NAD-dependent 65-kDa Fe-hydrogenase; 6, acetate:succinate CoA-transferase; 7, succinate thiokinase.

dehydrogenase components from mitochondrial complex I fills important gaps in the redox balance of hydrogenosome metabolism^{22,23}. The enzyme can replenish the NAD⁺ pool by reoxidizing NADH produced by the hydrogenosomal malic enzyme (Fig. 5). It can also catalyse the previously elusive NADH:ferredoxin oxidoreductase activity, to provide an alternative source of reducing power to that provided by pyruvate:ferredoxin oxidoreductase¹. Its suspected interactions (Fig. 5) with the *Trichomonas* 65-kDa Fe-hydrogenase, which contains similar domains to the 75-kDa subunit of mitochondrial complex I (ref. 7) and to bacterial NAD-reducing hydrogenase²⁴, have yet to be demonstrated experimentally.

Our findings and previously published data^{9–12} indicate that mitochondria and *Trichomonas* hydrogenosomes are homologues *sensu* Owen²⁵, meaning 'the same organ [here organelle] in different animals under every variety of form and function'. This hypothesis explains the shared similarities between the two organelles most simply and therefore is the one that must be rejected before alternative models are deemed necessary. Furthermore, the hypothesis⁶ that the *Trichomonas* hydrogenosome could be the product of a second invasion of eukaryotic cells by an anaerobic bacterium does not account for the observation, unless further invasions are posited, that hydrogenosomes also occur in fungi⁴ and diverse ciliate lineages²⁶. The 'atypical anaerobic metabolic enzymes', whose presence was used to underpin this hypothesis⁶, are also not unique to hydrogenosomes. A protein comprising a homologous pyruvate:ferredoxin oxidoreductase fused with NADPH-cytochrome P450 reductase occurs in *Euglena* mitochondria²⁷, and a homologous iron [Fe]-hydrogenase is found in the plastids of green algae and the cytosol of *Entamoeba* and *Giardia*⁴. By contrast, the presence of complex I components of mitochondrial ancestry in hydrogenosomes underscores their common endosymbiotic origin and uncovers a potential new route to hydrogen production in these anaerobic versions of mitochondria. □

Methods

Organisms, cultivation and cell fractionation

T. vaginalis strains T1 and G3 were maintained in Trypticase–yeast extract–maltose (TYM) medium with 10% heat-inactivated horse serum at 37 °C. Cytosolic and hydrogenosomal fractions were prepared as described¹⁰. Hydrogenosomes were isolated by isopycnic centrifugation on a 45% Percoll gradient¹⁰.

Identification of a NuoF-like gene in *T. vaginalis*

A clone identified in a *T. vaginalis* G3 EST library encoding a nearly complete 51-kDa subunit/NuoF homologue was used to isolate a full-length sequence (Tvh-47) from a *T. vaginalis* T1 λ ZAPII genomic library²⁸.

Additional components of complex I on the *T. vaginalis* genome

Published sequences of eukaryotic complex I subunits were used in tBlastn searches to identify a homologue of the 24-kDa subunit of complex I (NuoE) on the genome of *T. vaginalis* strain G3 (TIGR: <http://www.tigr.org/tdb/e2k1/tvg/>).

Phylogenetic analyses

The *Trichomonas* NuoF and NuoE amino-acid sequences were aligned to a broad sample of homologous sequences from the NCBI. Bayesian phylogenetic analyses used MrBayes 3.0B4 (ref. 29) for amino acid alignments, and p4 (ref. 18) for alignments of recoded amino acids. In the latter, the amino acids were recoded into six categories corresponding to the PAM matrix (and most other matrices) as follows: (1) ASTGP, (2) DNEQ, (3) RKH, (4) MVIL, (5) FYW and (6) C. This allowed the use of a 6 × 6 general time-reversible rate matrix with free parameters rather than a fixed empirical matrix, composition and among-site rate variation parameters were also free.

Recombinant protein and antibody preparation

The complete Tvh-47 plus an amino-terminal His tag was expressed in *Escherichia coli* and purified under denaturing conditions on Ni(II)-nitrilotriacetate resin (Qiagen). A rabbit polyclonal antibody was raised against the recombinant protein as described³⁰.

T. vaginalis transformation

The plasmid TagVag-47 was constructed by amplifying the Tvh-47 ORF and ligating it into pCR2.1-TOPO plasmid (Invitrogen). The di-haemagglutinin ((HA)₂) epitope tag was amplified from Tviscs2-(HA)₂ (ref. 10) and cloned into pCR2.1-TOPO. A fragment containing Tvh-47 and (HA)₂ was then subcloned into pAPlac. The plasmid was digested by SacII–NdeI and the 5' untranslated region of AP65-1 was replaced by 340 base pairs of the 5' untranslated region of *T. vaginalis* α-succinyl CoA synthetase. *T. vaginalis* cells were electroporated and selected in TYM medium supplemented with G418 (Sigma)¹⁰.

Enzyme assays and purification of NADH dehydrogenase

For details of these procedures see Supplementary Information.

Recombinant ferredoxins

Recombinant *T. vaginalis* 2Fe–2S ferredoxin was produced as described¹⁰. Recombinant *Saccharomyces cerevisiae* mitochondrial 2Fe–2S ferredoxin, fused with a polyhistidine tag, was provided by A. Dancis (University of Pennsylvania).

Identification of peptides by mass spectrometry

Tryptic peptide fragments of purified NADH dehydrogenase subunits were sequenced by quadrupole time-of-flight mass spectrometry (Q-TOF) using an ULTIMA API nanospray mass spectrometer after separation by reverse-phase high-performance liquid chromatography (CapLC; Waters).

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A candidate NAD⁺ transporter in an intracellular bacterial symbiont related to Chlamydiae

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Bacteria living within eukaryotic cells can be essential for the survival or reproduction of the host^{1,2} but in other cases are among the most successful pathogens^{3,4}. Environmental Chlamydiae, including strain UWE25, thrive as obligate intracellular symbionts within protozoa; are recently discovered relatives of major bacterial pathogens of humans; and also infect human cells^{4–7}. Genome analysis of UWE25 predicted that this symbiont is unable to synthesize the universal electron carrier nicotinamide adenine dinucleotide (NAD⁺)⁷. Compensation of limited biosynthetic capacity in intracellular bacteria is usually achieved

by import of primary metabolites^{8–11}. Here, we report the identification of a candidate transporter protein from UWE25 that is highly specific for import of NAD⁺ when synthesized heterologously in *Escherichia coli*. The discovery of this candidate NAD⁺/ADP exchanger demonstrates that intact NAD⁺ molecules can be transported through cytoplasmic membranes. This protein acts together with a newly discovered nucleotide transporter and an ATP/ADP translocase¹², and allows UWE25 to exploit its host cell by means of a sophisticated metabolic parasitism.

The genome sequence of UWE25 (ref. 7) contains five genes (*ntt1* to *ntt5*) with sequence homology to functionally characterized ATP/ADP translocases or nucleotide transporters (NTT) of other bacteria¹³. It is tempting to speculate that these genes encode transporters essential for survival of UWE25 by linking symbiont and host metabolism. However, the specific properties of these putative transporters can not be predicted from sequence analyses¹⁴ but require heterologous expression in *E. coli*, leading to biochemical properties similar to the authentic carriers in their native membranes^{14–18}. Heterologous expression of NTT1 of UWE25 and its biochemical characterization demonstrated that this transporter is used for energy parasitism by enabling uptake of host cell ATP in exchange with ADP¹².

In order to investigate whether NTT-type carriers of UWE25 also compensate for lack of nucleotide and NAD⁺ biosynthesis, two further NTTs were functionally characterized in this study. NTT2 (516 amino acids) exhibits highest sequence identity (47%) to the ATP/ADP translocase CntNTT1 from *Chlamydia trachomatis*. NTT4 (431 amino acids) shows highest sequence identity (22%) to the plastidic ATP/ADP transporter AtNTT1 from *Arabidopsis thaliana*, exhibits nine predicted transmembrane domains, and lacks, in contrast to NTT2, several amino acid residues critical for nucleotide transport (Supplementary Fig. 1)¹⁹.

Heterologously synthesized NTT2 transports ATP, GTP and UTP at high rates, but ADP import is much lower. This transport is specific because uninduced *E. coli* cells do not import labelled compounds (Supplementary Fig. 2a). This import closely resembles the characteristics of CntNTT2, a unidirectional nucleotide transporter from *C. trachomatis*¹⁴. The functional similarity of CntNTT2 and NTT2 is interesting, because the amino acid sequence of NTT2 exhibits the highest similarity to the ATP/ADP translocase CntNTT1 rather than CntNTT2 (Supplementary Fig. 1). Moreover, phylogenetic analysis indicates a closer relationship of UWE25 NTT2 to ATP/ADP translocases than to the chlamydial nucleoside-triphosphat uniporters (Supplementary Fig. 3). This observation illustrates that it is impossible to make reliable predictions on the transport characteristics of NTT-type proteins solely on the basis of amino acid sequence identities.

Heterologous expression of NTT4 demonstrated that this transporter does not accept nucleoside triphosphates, but instead catalyses ADP uptake. Uptake of radioactively labelled nucleotides by NTT4 depends upon induction of the corresponding gene (Supplementary Fig. 2b). Such a clear substrate preference for a nucleoside diphosphate, rather than nucleoside triphosphates, is unusual and prompted further transport analyses in the presence of putative inhibitors. Notably, the presence of unlabelled NAD⁺ almost abolished [³²P]-ADP uptake, as did NADH and ADP, whereas NADP⁺ and NADPH inhibited [³²P]-ADP uptake only slightly (Fig. 1a).

In order to reveal the properties of NTT4 in more detail, we analysed the transport of [adenylate-³²P]-labelled NAD⁺. NTT4-expressing *E. coli* imported [³²P]-NAD⁺ at a rate of about 80 nmol mg^{−1} of protein per hour, and the simultaneous presence of unlabelled NAD⁺ (fivefold excess) inhibited uptake to less than 20% of the control rate (Fig. 1b). A high substrate specificity of NTT4 is further underlined by the fact that the substrate acceptance is determined in various domains of the NAD⁺ molecule, as

NADP⁺, nicotinic acid adenine dinucleotide (NAAD⁺), ATP, ADP and AMP failed to inhibit [³²P]-NAD⁺ uptake (Fig. 1b, and data not shown). Furthermore, NAD⁺ uptake by NTT4 occurs at a high apparent affinity (Michaelis constant (K_m) of 15 μ M, Fig. 1c).

With one exception¹⁴, all members of the NTT family perform a counter exchange mode of transport¹³. To analyse whether NTT4 of strain UWE25 also performs a counter exchange, we performed experiments with NTT4-expressing *E. coli* preloaded either with labelled ADP or NAD⁺ in the presence or absence of putative counter exchange substrates. NTT4-expressing *E. coli* preloaded with [³²P]-labelled ADP released internal radioactivity (mainly as [³²P]-P_i, where P_i indicates inorganic phosphate) already in the presence of phosphate-buffer medium only (Fig. 2a, c). This

observation has been made previously after expression of a plastidic ATP/ADP translocase from *Arabidopsis* in *E. coli*¹⁷, and suggests that an endogenous *E. coli* transporter is responsible for P_i export. However, when ADP, NAD⁺ or NADH were added to the phosphate buffer medium as counter exchange substrates, export of radioactivity was further stimulated, and ADP was recognized as the major export compound (Fig. 2a, c). This observation is clearly indicative of a counter exchange mode of transport catalysed by NTT4. *Escherichia coli* that synthesized NTT4 and were preloaded with labelled NAD⁺ hardly released radioactivity when re-suspended in either P_i buffer, or in ADP-containing buffer (Fig. 2b). In contrast, the presence of both NAD⁺ or NADH in the medium provoked a rapid export of internal label, further supporting the counter exchange mode of the NTT4 transporter (Fig. 2b). Although heterologous synthesis of NTT4 in *E. coli* enabled us to identify fundamental transport properties of this carrier under conditions closely resembling those in UWE25, analyses of some of its biochemical properties (such as determination of K_m for the counter exchange substrates) will have to await reconstitution of pure carrier protein in proteoliposomes.

On the basis of the direct uptake data (Fig. 1b, c) and the back exchange results (Fig. 2b) it seems possible that both NAD⁺ and NADH represent counter exchange substrates for NTT4. However, as cellular NAD⁺ levels exceed those of NADH several-hundred-fold^{20,21}, binding of NADH to NTT4 will only occur very rarely, excluding the importance of NAD⁺/NADH exchange under *in vivo* conditions. Whether NAD⁺ or ADP will serve as counter exchange substrate for NAD⁺ uptake will depend on the intracellular concentration ratio of NAD⁺ to ADP in strain UWE25. On the outer substrate-binding side, exposed to the outer bacterial surface, NTT4 has a much higher affinity for NAD⁺ than for ADP (Fig. 1). Assuming that this also holds true for the inner binding side of this transporter, NTT4 catalyses a NAD_{in}⁺/ADP_{out} transport if the NAD⁺ to ADP ratio is low in strain UWE25 (as seen in NTT4-expressing *E. coli*; Fig. 2a, c). Such a scenario probably occurs in recently divided UWE25 cells. During growth of these cells the cytoplasmic nucleotide pool will be diluted. The ADP level will become replenished by the action of NTT2 and subsequent use of imported ATP. The NAD⁺ level remains low until bacterial ADP is used for NTT4-catalysed NAD⁺ import from the host cell. As soon as the NAD⁺ pool of UWE25 is filled, NAD⁺ inhibits ADP export and NTT4 runs in a NAD⁺/NAD⁺ idling cycle (as seen in NTT4-expressing *E. coli*; Fig. 2b). This mode of transport does not provide additional NAD⁺ to UWE25 nor does it represent any disadvantage, because NAD⁺ homeostasis is not negatively affected.

NTT4 represents the first member of the NTT protein family that has been found to counter exchange structurally very different substrates¹³, and transport of intact NAD⁺ molecules across biological membranes has not been described previously²². However, several bacteria are able to hydrolyse NAD⁺ extracellularly and to make use of one or both of the resulting products. For example, *Rickettsia prowazekii* is unable to import the hydrolysis product nicotinamide mononucleotide, but takes up the produced AMP moiety, which is partly used for intracellular re-synthesis of NAD⁺ (ref. 11). In contrast to that mechanism, there are several lines of evidence indicating that NTT4 from UWE25 is the first recognized transporter for intact NAD⁺ across membranes. First, no labelled products other than NAD⁺ are detectable in cellular extracts from NTT4-expressing *E. coli* incubated with extracellular [³²P]-NAD⁺ (Fig. 1c, right inset). In contrast, labelled NAD⁺ and labelled AMP were both observed in cellular extracts of *R. prowazekii* after exposure to [³²P]-NAD⁺ (ref. 11). Second, in contrast to the system in *Rickettsia*, adenine nucleotides do not markedly inhibit NAD⁺ uptake in NTT4-expressing *E. coli* (Fig. 1b). Finally, *E. coli* cells expressing NTT4 and preloaded with [³²P]-NAD⁺ export [³²P]-NAD⁺ if NAD⁺ or NADH are present as counter exchange substrates (Fig. 2b).

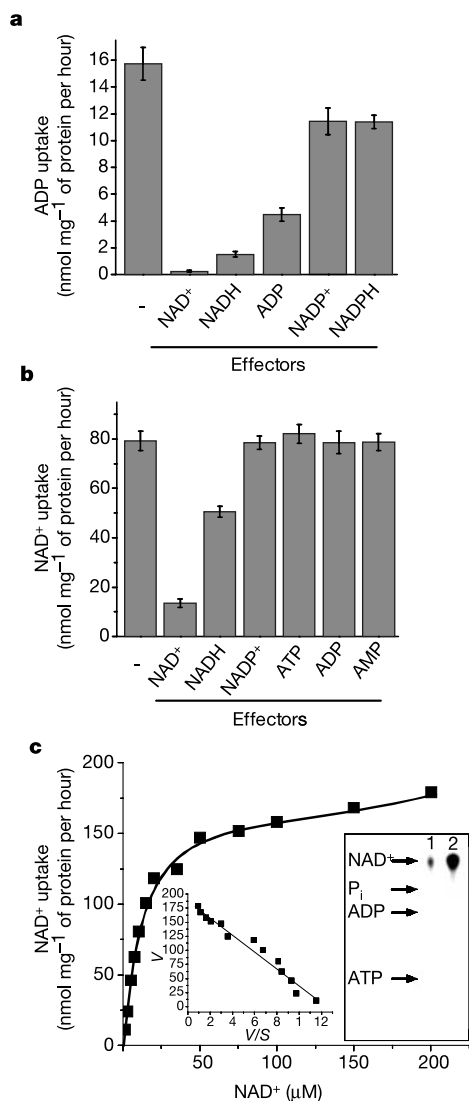


Figure 1 Effect of metabolites on ADP or NAD⁺ uptake by NTT4 and substrate saturation analysis. **a**, Effect of metabolites on ADP uptake. A total of 50 μ M [³²P]-ADP was used; inhibitors were present at 500 μ M ($n = 3$; mean $\pm$ s.e.m.). The affinity constant K_m of NTT4 for ADP is $275 \pm 11 \mu$ M ($\pm$ standard error), V_{max} is 67.3 ± 5.3 nmol mg⁻¹ of protein per hour ($\pm$ standard error) (data not shown). **b**, Effect of metabolites on NAD⁺ import. A total of 10 μ M [³²P]-NAD⁺ was present; inhibitors were applied at 50 μ M ($n = 3$; mean $\pm$ s.e.m.). No [³²P]-NAD⁺ uptake was measured using uninduced *E. coli* cells (data not shown). **c**, Substrate saturation of NAD⁺ uptake. The left inset represents an Eadie-Hofstee analysis, indicating a K_m NAD⁺ of 15.0 μ M, and a V_{max} of 188.8 nmol mg⁻¹ of protein per hour ($n = 3$; mean; standard error less than 8%). The right inset shows thin-layer chromatography of labelled compounds present in NTT4-expressing *E. coli* incubated with [³²P]-NAD⁺. Lane 1, cellular extracts from *E. coli* synthesizing NTT4 and incubated with [³²P]-NAD⁺ (1 min); lane 2, [³²P]-NAD⁺ standard.

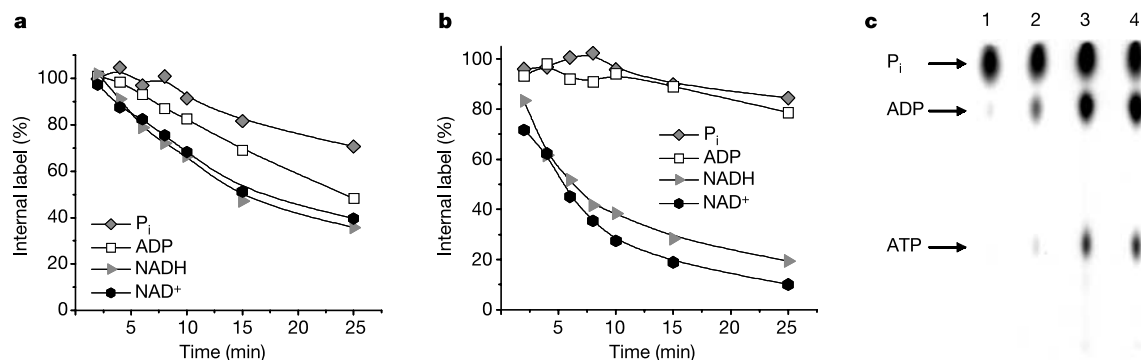


Figure 2 Back exchange properties of NTT4. **a**, *Escherichia coli* synthesizing NTT4 preloaded with labelled [³²P]-ADP (10 μ M, 2 min). After removal of external radioactivity, export of label was induced by re-suspension in phosphate buffer, with or without effector (1 mM). **b**, *Escherichia coli* synthesizing NTT4 preloaded with [³²P]-NAD⁺ (2 μ M, 2 min).

Cells were treated as stated above. **c**, Thin-layer chromatographic identification of labelled metabolites exported from NTT4-expressing *E. coli* preloaded with [³²P]-ADP (10 μ M, 2 min). Export was allowed for 5 min. Export in P_i buffer (lane 1), in the presence of ADP (200 μ M; lane 2), NAD⁺ (200 μ M, lane 3), or NADH (200 μ M, lane 4).

On the basis of the functional characteristics of NTT1, NTT2 and NTT4 of UWE25 and the simultaneous transcription of the corresponding genes during multiplication of UWE25 within amoebae (Fig. 3), a model for metabolic interaction of UWE25 with its protozoan host is postulated (Fig. 4). UWE25 uses the ATP/ADP translocase NTT1 to import ATP from the host cytosol in

counter exchange to bacterial ADP. This energy parasitism connects the endosymbiont to the energy status of the host cell¹². The NAD⁺ transporter NTT4 is used by UWE25 to get access to host cell NAD⁺ (which can be converted to NADP by the UWE25 NAD⁺ kinase) to compensate for its inability to synthesize these compounds⁷. During this process the endosymbiont loses ADP, which is replenished by the import of ATP via the nucleotide transporter NTT2. In addition, this transporter serves the symbiont for net acquisition of other nucleotides, which cannot be generated *de novo* by UWE25 (ref. 7).

The discovery of the first candidate NAD⁺ membrane transporter in the chlamydial endosymbiont UWE25 shows that our current perception of metabolic links between eukaryotes and prokaryotes is far from complete. Because pathogenic Chlamydiae also lack the ability to synthesize NAD⁺ it seems likely that these highly successful bacterial pathogens exploit a similar mechanism for thriving in eukaryotic cells, although no respective homologous genes have yet been identified (Supplementary Table 1). If this hypothesis holds true, NAD⁺ transporters do not only extend our understanding of general chlamydial biology, but they may also represent promising drug targets for anti-chlamydial therapy. □

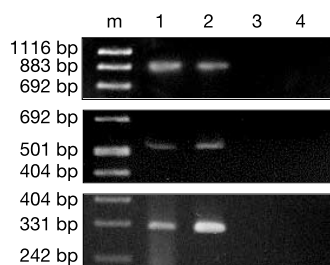


Figure 3 Transcriptional analysis of *ntt1* (top) *ntt2* (middle) and *ntt4* (bottom) of strain UWE25 during multiplication within *Acanthamoeba*. Lane 1, amplification products of cDNA synthesized from whole RNA from amoebae harbouring UWE25; lane 2, PCR positive controls using DNA from purified UWE25 elementary bodies; lane 3, PCR negative controls (no cDNA added); lane 4, PCR using whole RNA from amoebae containing UWE25 (control for the absence of DNA in the RNA preparation, no reverse transcriptase added); m, molecular-mass marker.

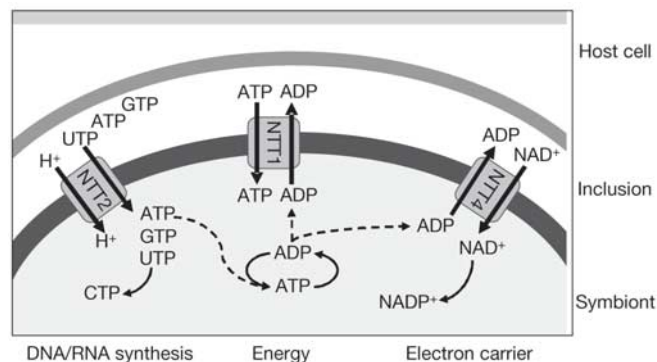


Figure 4 Model for metabolic interactions between UWE25 and its amoeba host. The concerted action of NTT transporters allows for a novel type of bacterial parasitism by importing DNA/RNA synthesis precursors (nucleotides), energy (ATP) and the electron carrier NAD⁺ from the host cell. According to its genome sequence, UWE25 is capable of generating NADP from NAD⁺, and CTP from UTP⁷. This model implies that presence of *ntt* transcripts correlates with transporter protein expression and activity.

Methods

Identification of NTT genes and transcription analysis

The complete genome sequencing of UWE25 revealed the presence of five genes encoding proteins with homology to the NTT transporter family, which were named *ntt1*, *ntt2*, *ntt3*, *ntt4* and *ntt5*, respectively⁷ (<http://webclu.bio.wzw.tum.de/genre/proj/uwe25/>). Transmembrane topology prediction analysis was performed using TMpred²³. Comparative sequence analysis of NTT proteins was performed using ClustalX²⁴ and the ARB software package²⁵.

UWE25 was grown in the host *Acanthamoeba* sp. UWC1 (ref. 26) as described previously¹². For transcriptional analysis amoebae harbouring UWE25 were collected by centrifugation (2,350g, 5 min, 4 °C), re-suspended in TRIzol (Invitrogen Life Technologies) and immediately homogenized using the Bead-Beater Fast Prep FP120 Instrument (BIO 101). Whole RNA purification was performed according to the recommendations of the manufacturer, followed by a DNase treatment using DNase I. A control polymerase chain reaction (PCR) using 16S ribosomal RNA gene-targeted primers was performed to ensure that the RNA preparation was free of DNA, and 1 μ g of RNA was used for complementary DNA synthesis using the RevertAid first strand cDNA synthesis kit (MBI-Fermentas) and a gene-specific primer as suggested by the manufacturer. cDNA was subsequently used as template in standard PCR reactions. Negative controls (no cDNA added) were included in all PCR reactions. A standard PCR cycling programme with 40 cycles and an annealing temperature of 54 °C was used for the amplification of the *ntt1*, *ntt2* and *ntt4* cDNA. Primers targeting an 844-base pair (bp) fragment of *ntt1* (ref. 12), a 511-bp fragment of *ntt4* (forward primer: 5'-TCATAGGATTAGCTCTGC-3'; reverse primer: 5'-AACAACTACCATGGC-3'), or a 304-bp fragment of *ntt2* (forward primer: 5'-TGCTGCCGATGATGTTGATGC-3'; reverse primer: 5'-GTAATCGCATAGTTGTAGAGG-3') were used for cDNA synthesis and PCR reactions, respectively. Amplification products were sequenced to ensure that amplification was specific. Experiments were performed in triplicate.

NTT2- and NTT4-mediated substrate uptake by *E. coli*

For two of the four newly identified NTT isoforms in UWE25 (NTT2 and NTT4) we were able to optimize heterologous expression in *E. coli* in order to elucidate their biochemical

properties. Simultaneous isolation of DNA from amoebae and their bacterial endosymbionts was performed using the FastDNA-Kit (BIO 101) according to the protocols recommended by the manufacturer. *ntt2* and *ntt4* were amplified with the Extensor hi-fidelity PCR enzyme mix (ABgene) using forward primers introducing a *NdeI* restriction site instead of the start codon (5'-CAGCATATGCTCAACAAGATCAGAGTTTGGT-3' for *ntt2*, and 5'-CAGGGCCATATGAGTAAAAACAACAGGTA-3' for *ntt4*) and reverse primers containing a *BamHI* restriction site after the stop codon (5'-TTGGGATCCTTAAGATGCAGCTTTAAGGGATG-3' for *ntt2*, and 5'-CTAGCAGGATCCTTATTTTATAAAAGCGCT-3' for *ntt4*). The resulting amplification products were digested with *BamHI* and *NdeI*, purified and inserted into the expression vector pET16b (Novagen). The newly constructed plasmids (pNTT2, pNTT4) were transformed into and maintained in *E. coli* XL1Blue (Stratagene). Identity of the cloned genes was checked by sequencing.

To analyse transport specificity of NTT2 and NTT4, 100 µl of induced *E. coli* cells harbouring either the plasmid pNTT2 or pNTT4 were added to 100 µl of incubation medium (50 mM P_i buffer medium, pH 7.2) containing the [³²P]-labelled nucleotides [α-³²P]-ATP, -GTP, -UTP, or [adenylate-³²P]-NAD⁺ (NEN). [α-³²P]-labelled ADP was synthesized as described previously¹⁶. Specific activities of all nucleotides ranged between 80 and 250 µCi mmol⁻¹. Uptake of nucleotides was carried out at 30 °C¹⁴ and terminated after the indicated time periods by transfer of the cells onto a 0.45-µm nitrocellulose filter (Schleicher and Schüll), pre-wetted with incubation medium and set under vacuum. Cells were immediately washed by addition of 2 × 1.5 ml ice-cold incubation medium¹⁶. The filters were subsequently transferred into 20-ml scintillation vessels, containing 5 ml water, and radioactivity in these samples was quantified in a scintillation counter (Canberra-Packard Tricarb 2500).

Back-exchange studies and thin-layer chromatography

Escherichia coli cells synthesizing either NTT2 or NTT4 from UWE25 were incubated for an appropriate time span in phosphate buffer medium containing radioactively labelled ADP or NAD⁺. Subsequently, cells were collected by centrifugation and re-suspended in ice-cold phosphate buffer medium to remove non-imported radioactivity. After a second centrifugation step cells were re-suspended in phosphate buffer, with or without the indicated counter exchange substrates. For thin-layer chromatography *E. coli* cells were collected by centrifugation and an aliquot of the supernatant was loaded onto a 0.5-mm polyethylene amine-cellulose thin-layer chromatography plate and dried with a fan¹⁶. Separation of adenine nucleotides and NAD⁺ was conducted according to a standard protocol^{16,27}. RF values of radioactively labelled compounds were determined after radioautography and corresponded to RF values of both unlabelled nucleotides visualized under ultraviolet light and radioactively labelled standards¹⁴.

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Haemangioblast commitment is initiated in the primitive streak of the mouse embryo

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Haematopoietic and vascular cells are thought to arise from a common progenitor called the haemangioblast. Support for this concept has been provided by embryonic stem (ES) cell differentiation studies that identified the blast colony-forming cell (BL-CFC), a progenitor with both haematopoietic and vascular potential^{1,2}. Using conditions that support the growth of BL-CFCs, we identify comparable progenitors that can form blast cell colonies (displaying haematopoietic and vascular potential) in gastrulating mouse embryos. Cell mixing and limiting dilution analyses provide evidence that these colonies are clonal, indicating that they develop from a progenitor with haemangioblast potential. Embryo-derived haemangioblasts are first detected at the mid-streak stage of gastrulation and peak in number during the neural plate stage. Analysis of embryos carrying complementary DNA of the green fluorescent protein targeted to the *brachyury* locus demonstrates that the haemangioblast is a subpopulation of mesoderm that co-expresses *brachyury* (also known as *T*) and *Flk-1* (also known as *Kdr*). Detailed mapping studies reveal that haemangioblasts are found at highest frequency in the posterior region of the primitive streak, indicating that initial stages of haematopoietic and vascular commitment occur before blood island development in the yolk sac.

To determine whether haematopoietic and vascular lineages in the early mouse embryo develop from a haemangioblast, cells from pools of late streak to neural plate stage concepti (presumptive yolk sac and embryo proper, embryonic day (E) 7.0–7.5) were plated under conditions similar to those that support the development of embryoid body-derived BL-CFCs. Colonies with the morphology of the BL-CFC-derived blast colonies developed within 3–4 days of

culture¹ (Fig. 1a, b). Molecular analysis of these embryo-derived colonies indicated that they expressed genes associated with haematopoietic and vascular development including *Flk-1* (refs 3–5), *Scl*^{6,7} (also known as *Tal1*), VE-cadherin⁸ (*Cdh5*), *Gata1* (refs 7, 9), β H1 globin (*Hbb-bh1*) and β -major globin (*Hbb-b1*), with patterns similar to those found in embryoid body-derived blast colonies^{2,10} (Fig. 1c). The lack of expression of the mesodermal gene *brachyury*¹¹

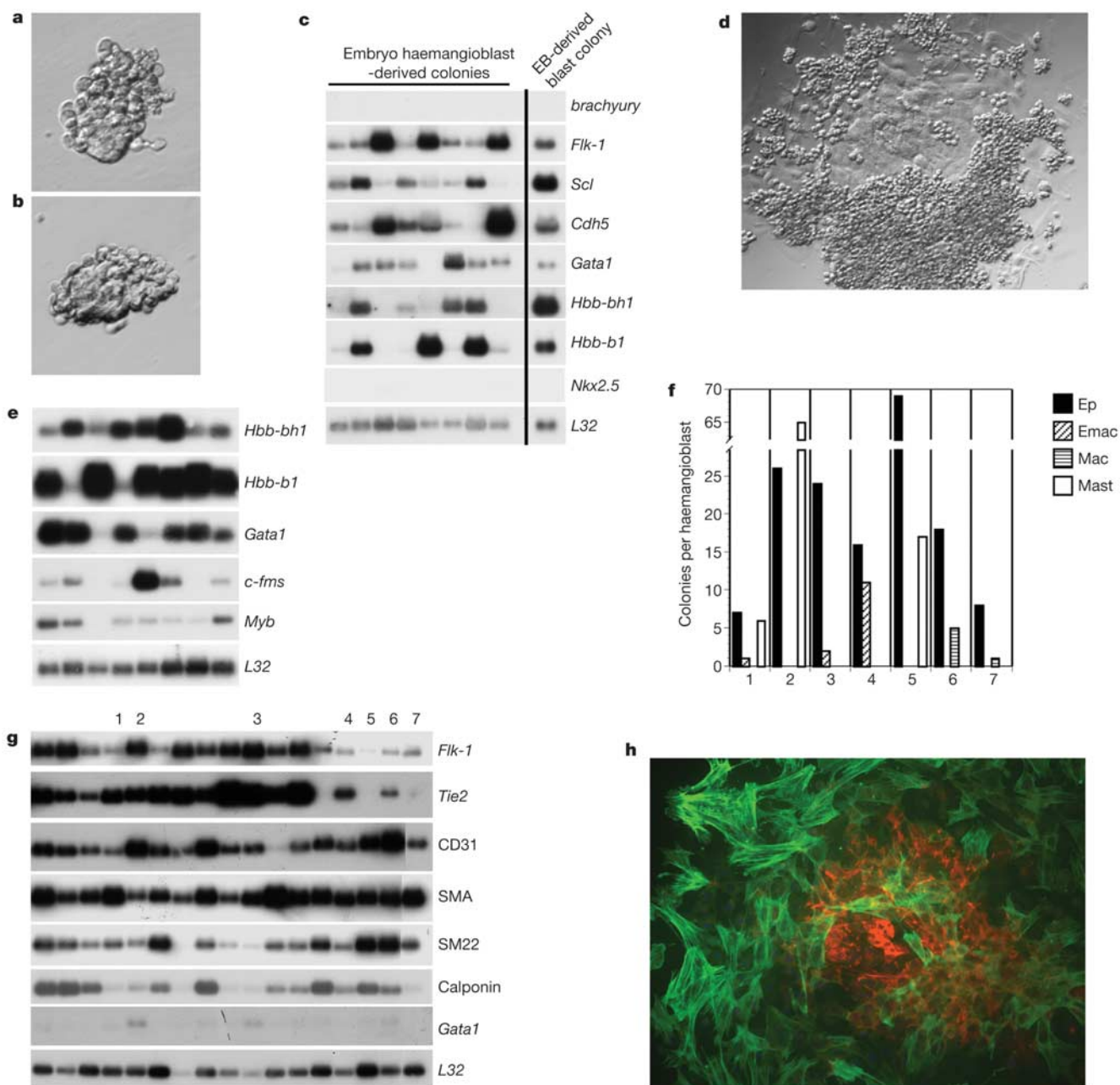


Figure 1 Characteristics and cell lineage potentials of the mouse haemangioblast. **a**, A haemangioblast-derived colony from an E7.5 embryo. **b**, A blast colony from an E3.25 embryoid body. **c**, Gene expression analysis of eight haemangioblast-derived colonies contrasted with that of a representative blast colony derived from an embryoid body (EB). *L32* encodes a component of the 60S ribosomal protein, and was used as a control for the amount of cDNA loaded in each lane. **d**, Adherent and non-adherent cells resulting from the expansion of a haemangioblast-derived colony in liquid medium. Original magnification $\times 200$. **e**, Expression analysis of non-adherent cells from eight expanded haemangioblast-derived colonies. **f**, The haematopoietic progenitor potential of

the non-adherent cells from seven expanded haemangioblast-derived colonies. Ep, primitive erythroid; mac, macrophage; emac, bipotential erythroid macrophage; mast, mast cell. **g**, Gene expression analysis of adherent cells from expanded haemangioblast-derived colonies. The labelled lanes represent adherent cells obtained from the same colonies as the non-adherent cells used for the haematopoietic progenitor analysis shown in **f**. SMA, smooth muscle actin. **h**, The adherent cells from a haemangioblast-derived colony stained for the expression of smooth muscle actin (green, vascular smooth muscle) and CD31 (red, endothelial cells). Original magnification $\times 200$.

shows that they had progressed beyond the mesoderm stage of development, and the finding that they did not express the cardiac-specific gene *Nkx2.5* (ref. 12) indicates that they were not undergoing cardiac development. Cardiac mesoderm derived from ES cells will form colonies that express *Nkx2.5* in the blast cell colony conditions, demonstrating that these conditions are not inhibitory to the development of this lineage (S. Kattman and G.K., unpublished observations).

When plated in liquid culture on a thin layer of matrigel in the presence of haematopoietic and vascular cytokines, individual embryo-derived colonies generated both adherent and non-adherent cells (Fig. 1d). The non-adherent population expressed the haematopoietic markers $\beta H1$ and β -major globin, as well as *Gata1*, *c-fms* (also known as *Csf1r*) and *Myb* (Fig. 1e), indicative of the presence of primitive erythroid, macrophage and immature haematopoietic cells¹³. When cultured in methylcellulose, the non-adherent populations of single expanded colonies generated both primitive erythroid and definitive haematopoietic (macrophage and mast) colonies, confirming the potential predicted by the molecular analysis (Fig. 1f). From a total of 118 replated colonies in eight separate experiments, 73 generated haematopoietic progenitors with the following potentials: 37% restricted to primitive erythroid potential, 47% with primitive erythroid and definitive potential and 16% with only definitive potential. These findings indicate that there is heterogeneity in the haematopoietic potential of these colonies.

The adherent cells from the expanded colonies expressed genes representing endothelial and vascular smooth muscle development (Fig. 1g), including *Flk-1*, *Tie2* and CD31 (endothelial; *Pecam1*), and smooth muscle actin, SM22 (*Tagln*) and calponin (vascular smooth muscle)^{14,15}. Endothelial and vascular smooth muscle cell lineages are known to develop from the blast colony^{1,16}. Immunostaining of the adherent population generated from single colonies demonstrated the presence of a cluster of CD31⁺ endothelial cells, surrounded by an outgrowth of larger vascular smooth muscle cells expressing smooth muscle actin (Fig. 1h). Contracting cells indicative of cardiac development have not been observed in the expanded cultures, consistent with the above expression analysis indicating that this other mesoderm derivative does not develop from the blast colonies. The conditions used for expansion will support contracting cells from embryoid body-derived cardiac mesoderm (data not shown), demonstrating that the conditions are not inhibitory to this population. Taken together, these findings demonstrate that the embryo-derived colonies display primitive and definitive haematopoietic, endothelial and vascular smooth muscle potential and in this regard are similar, if not identical, to the BL-CFC colonies derived from embryoid bodies^{1,16}.

As an initial assessment of the clonality of the embryo-derived colonies, cells from embryos carrying either a neomycin or hygromycin transgene were mixed and plated under conditions appropriate for haemangioblast development. The non-adherent (haematopoietic) and adherent (vascular) populations of 38 haemangioblast-derived colonies expanded in liquid culture were harvested for genomic DNA and analysed for the neomycin and hygromycin genes by polymerase chain reaction (PCR, Fig. 2a). Twenty colonies gave non-adherent and adherent cell populations that carried the neomycin transgene only, whereas 17 colonies gave cell populations that carried the hygromycin transgene only. In one colony, neomycin and hygromycin DNA was found in both adherent and non-adherent cell populations from most of the embryo haemangioblast-derived colonies strongly suggests that they originate from a single cell. In addition, these findings argue against the occurrence of cell fusion in our cultures as this would have resulted in a higher frequency of haematopoietic and vascular cells containing both neomycin and hygromycin genes. Given the haematopoietic and vascular potential of these colonies,

the cell that gives rise to these colonies can be considered the haemangioblast.

To determine when haemangioblasts are generated during early embryogenesis, a kinetic analysis was performed on embryos ranging from the early streak to the head fold stage of development (E6.75 to E8.0.) (Fig. 2b). No haemangioblast-derived colonies were detected in the early streak stage embryos. Approximately 28% of the mid-streak embryos contain haemangioblasts, whereas 59% of those at the late streak/early neural plate stage and 88% of the embryos at neural plate stage were found to contain these progenitors. Most of the embryos at the late streak/early neural plate and neural plate stages were found to have between one and five haemangioblasts per embryo, but some had more than ten (the maximum number found was 33). Haemangioblast activity seems to decline after the neural plate stage as only 47% of those at the

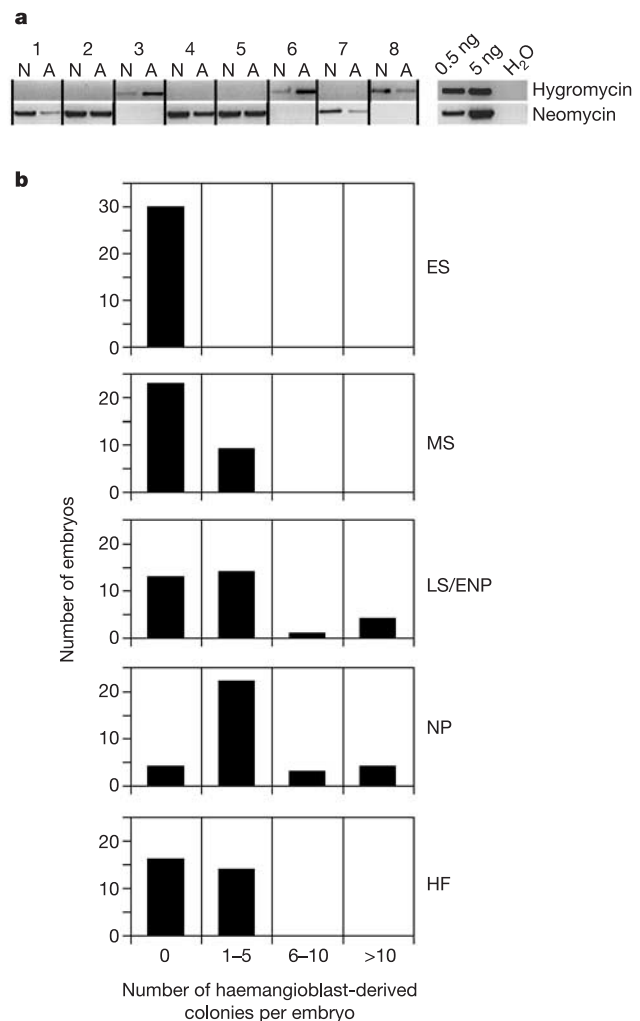


Figure 2 Clonality of haemangioblast-derived colonies and kinetics of haemangioblast development. **a**, PCR analysis of the non-adherent (N) or adherent (A) outgrowths from eight haemangioblast-derived colonies generated from a mixture of E7.5 embryos carrying either the neomycin or hygromycin gene. The controls represent 0.5 ng and 5 ng of genomic cDNA from embryonic stem cells containing either the neomycin or hygromycin gene. **b**, The haemangioblast potential of 30–33 embryos analysed at the indicated stages: ES, early streak (30 embryos); MS, mid-streak (32 embryos); LS/ENP, late streak/early neural plate (32 embryos); NP, neural plate (33 embryos); HF, head fold (30 embryos).

head fold stage contain these progenitors. These findings indicate that haemangioblast development within the mouse embryo is a dynamic process that is initiated at the mid-streak stage, peaks at the late streak/early neural plate and neural plate stages, and sharply declines at the head fold stage. This pattern defines the haemangioblast stage, which represents a narrow developmental window spanning approximately 12–18 h of mouse gestation.

Recent studies using an ES cell line with green fluorescence protein (GFP) cDNA targeted to the *brachyury* locus demonstrated that the ES-derived BL-CFC expresses *Flk-1* and *brachyury*, and thus represents a subset of mesoderm undergoing commitment to the haemangioblast lineages¹⁷. To determine whether the embryo-derived haemangioblast represents a similar stage of development, embryos from transgenic mice generated using the GFP-Bry ES cells were used. GFP-Bry^{+/−} embryos (E7.5) express GFP in the primitive streak where *brachyury* is normally expressed¹⁸ (Fig. 3a). Flow cytometric analysis of pooled embryos (E7.5) from matings of GFP-Bry^{+/−} males and wild-type females revealed the presence of two GFP-expressing populations: GFP⁺Flk-1⁺ and GFP⁺Flk-1[−], comparable to the populations present in embryoid bodies that have been differentiated for 3.5 days¹⁷ (Fig. 3b). It is worth noting that the GFP-positive cells represent only 50% of the cells that express *brachyury*, as theoretically only half of the embryos are transgenic. Molecular analysis of the GFP-expressing populations showed that the GFP⁺Flk-1⁺ cells expressed *Flk-1*, *brachyury* and *Scl*. The GFP⁺Flk-1[−] cells did not express appreciable levels of *Flk-1* or *Scl*, but did express *brachyury* at levels higher than in the GFP⁺Flk-1⁺ fraction (Fig. 3c). Haemangioblasts were found at a higher frequency in the GFP⁺Flk-1⁺ population compared with the GFP⁺Flk-1[−] population (Fig. 3d). By using only GFP-Bry^{+/−} embryos, we found that as in the embryoid body studies, the GFP⁺Flk-1[−] population did not give rise to haemangioblast-derived colonies (data not shown). When the relative size of the

two GFP⁺ populations is taken into consideration, the GFP⁺Flk-1⁺ and GFP⁺Flk-1[−] fractions contain 75% and 25% of the total number of haemangioblasts assayed, respectively (Fig. 3e). These findings demonstrate that most embryo haemangioblasts co-express *Flk-1* and *brachyury*, similar to the embryoid body-derived BL-CFCs. These findings indicate that these progenitors probably represent a subset of mesoderm committed to the haematopoietic and vascular lineages. The activity observed in the GFP⁺Flk-1[−] fraction may represent progenitors that are already committed to the haemangioblast programme but do not yet express *Flk-1* protein on the cell surface. Maturation of these progenitors within the methylcellulose cultures to the stage at which they express cell surface *Flk-1* would enable them to respond to vascular endothelial growth factor (VEGF) and generate a typical haemangioblast-derived colony. The co-expression of *Flk-1* and *brachyury* can be considered to be the signature of the haemangioblast, because the expression patterns of these genes in embryoid bodies segregate rapidly after this developmental stage (M. Kennedy and G.K., unpublished observations).

The presence of haemangioblasts in the Flk-1[−] and GFP-expressing fractions enables us to isolate sufficient numbers of cells (free of non-mesodermal cells; that is, the GFP[−]Flk-1[−] fraction) for dose-response experiments that can further validate the clonal nature of the colonies. For this analysis, the total Flk-1⁺ and GFP⁺ cell populations (Flk-1⁺GFP[−], Flk-1⁺GFP⁺ and Flk-1[−]GFP⁺) from 59 to 69 embryos were isolated by sorting and cultured at different concentrations. As indicated in Fig. 3f, the number of colonies was directly proportional to the number of cells plated when compared on a logarithmic scale. The relationship was linear with a slope of 1, indicating that a single cell accounts for the growth of an individual colony.

Given that the haemangioblast represents a subpopulation of mesoderm, we wanted to define its location in the gastrulating

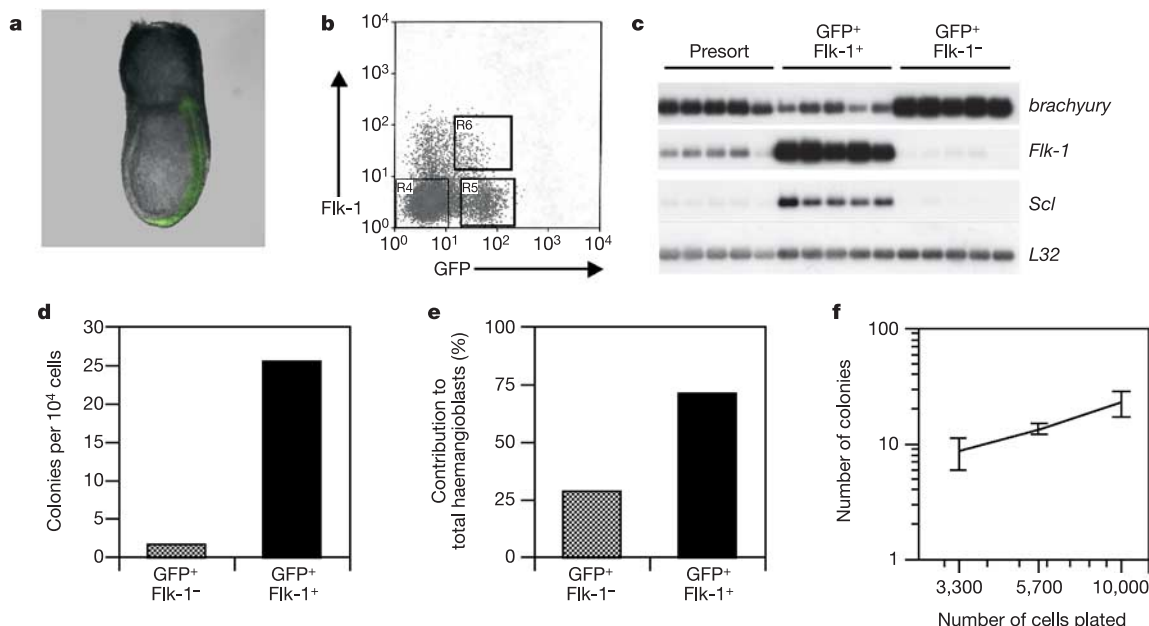


Figure 3 Isolation and characterization of GFP- and Flk-1-expressing cell populations from E7.5 GFP-Bry^{+/−} embryos. **a**, GFP expression in the primitive streak of an GFP-Bry^{+/−} embryo at the neural plate stage. **b**, Flow cytometric analysis of pooled embryos from matings of GFP-Bry^{+/−} males with wild-type females. The gates used to isolate the GFP⁺Flk-1[−] and GFP⁺Flk-1⁺ populations are indicated. **c**, Expression analysis of the GFP⁺Flk-1[−] and GFP⁺Flk-1⁺ sorted populations. Each lane represents 1,000 cells of the indicated populations. **d**, Frequency of haemangioblasts in the

GFP⁺Flk-1[−] and GFP⁺Flk-1⁺ populations. **e**, Percentage contribution of the GFP⁺Flk-1[−] and GFP⁺Flk-1⁺ populations to the haemangioblast potential of the total GFP population. **f**, Linear relationship of sorted Flk-1⁺ and/or GFP⁺ cells (includes GFP[−]Flk-1⁺, GFP⁺Flk-1⁺ and GFP⁺Flk-1[−] cells) from E7.5 embryos to number of haemangioblast-derived colonies. The experiment was performed three times. Results are presented as means ± s.e.m.

embryo. GFP-Bry^{+/−} embryos (E7.5) were dissected into fragments containing the yolk sac, posterior primitive streak, distal primitive streak, anterior region and lateral domains (Fig. 4a). The haemangioblast content of each portion of each embryo was determined. Most of the haemangioblasts (66%) were found in the posterior primitive streak, with little contribution from the distal primitive streak, the anterior region or the lateral domains. The yolk sac did contain some haemangioblasts, possibly reflecting progenitors that had migrated from the streak region and were undergoing differentiation. Gene expression analysis demonstrated the presence of *Flk-1* in the posterior primitive streak but not the distal primitive streak, consistent with the observed segregation of haemangioblast activity between these regions (Fig. 4b). The higher levels of the anterior marker cerberus-like (*Cer1*, refs 19, 20) in the distal compared to the proximal primitive streak and the presence of the posterior marker *Mesp1* (ref. 21) in the proximal but not in the distal primitive streak confirm the accuracy of the dissections. As expected, expression of *brachyury* was found in the primitive streak and yolk sac, indicating good separation of these regions from the lateral and anterior regions. *Gata1* expression is only found in the yolk sac, consistent with the initiation of hematopoiesis in this region.

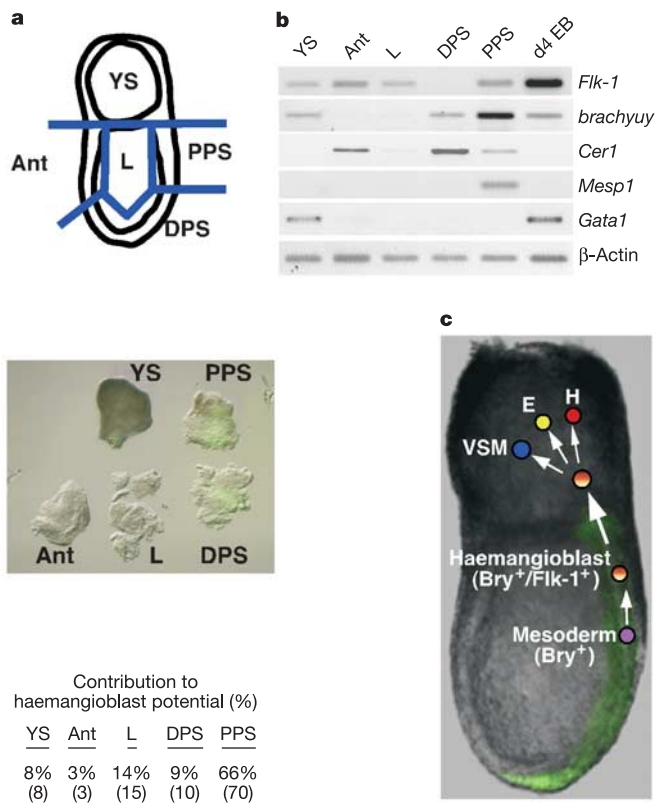


Figure 4 Localization of the haemangioblast in the embryo. **a**, GFP-Bry^{+/−} embryos at E7.5 were dissected into the yolk sac (YS), anterior (Ant), lateral (L), posterior primitive streak (PPS) and distal primitive streak (DPS) and cultured in haemangioblast conditions. The numbers of haemangioblast-derived colonies from fragments (shown in brackets) obtained from 23 embryos were summed (106 haemangioblasts total) and the percentage contribution each fragment made to the total haemangioblast potential of these embryos is indicated. **b**, Expression analysis on the dissected fragments. **c**, A model of haemangioblast development in the mouse embryo. The haemangioblast is a brachyury⁺ and Flk-1⁺ cell that arises in the primitive streak and migrates onto the yolk sac where it differentiates into haematopoietic (H), endothelial (E) and vascular smooth muscle (VSM) progenitor cells.

The localization of the haemangioblast to the posterior region of the primitive streak supports a model in which the earliest stages of haematopoietic and vascular commitment are initiated at this site, before migration of cells to the region of the presumptive yolk sac (Fig. 4c). The fact that relatively few progenitors are detected in the developing yolk sac suggests that the haemangioblasts rapidly differentiate into restricted haematopoietic and vascular progenitors as they egress from the streak. This model of haemangioblast development is consistent with previous findings indicating that one of the earliest deficiencies in *Flk-1* null embryos is the inability of primitive streak progenitors to migrate to the yolk sac²². These progenitors may be the equivalent of the haemangioblast identified in our study. The embryo haemangioblasts represent a transient progenitor population that is generated between the mid-streak and head fold stages of development. The observed heterogeneity in haemangioblast numbers between embryos probably reflects the dynamic nature of their development and indicates that these progenitors undergo rapid differentiation after their induction.

Lineage tracing studies have so far failed to identify a cell with haematopoietic and vascular potential in the early embryo²³. However, given the fact that the frequency of the haemangioblast is low (approximately 1 in 400 GFP⁺Flk-1⁺ cells, Fig. 3d), this progenitor could have been easily missed. Most evidence now suggests that haematopoiesis is initiated independently in the yolk sac and in the para-aortic splanchnopleura during early embryonic development and that it is different at each site^{24,25}. The yolk sac gives rise to the primitive erythroid lineage and a restricted subset of definitive haematopoietic progenitors but does not generate lymphoid progenitors and haematopoietic stem cells (HSCs) capable of long-term repopulation in recipient adult animals^{25,26}. The para-aortic splanchnopleura region supports the development of multilineage hematopoiesis, including lymphoid progenitors and HSCs, but does not give rise to primitive erythrocytes. The potential of the haemangioblast described here is consistent with the interpretation that it is the progenitor of yolk sac hematopoiesis. The fact that the highest number of haemangioblasts detected in a single embryo was 33 suggests that this number may be sufficient to establish this haematopoietic programme. As the para-aortic splanchnopleura represents a site of haematopoietic development in close proximity to blood vessels (dorsal aorta), it should also contain haemangioblasts. These haemangioblasts would represent the progenitors of the HSCs. Future studies using the GFP-Bry^{+/−} embryos should be able to elucidate whether haemangioblasts co-expressing *Flk-1* and *brachyury* also exist in the para-aortic splanchnopleura. Access to site-specific haemangioblasts would provide an opportunity to study how different haematopoietic programmes arise during development. □

Methods

Dissection and culture of mouse embryos

Three mouse mating schemes were used in this study. Outbred Swiss Webster females and males (Taconic Farms) were mated for the initial identification of the haemangioblast. To study the relationship of the haemangioblast to nascent mesoderm in the mouse embryo, we used a mesoderm model system where ES cells have GFP cDNA targeted to the *brachyury* locus¹⁷. Blastocysts were injected with GFP-Bry ES cells and the resulting chimaeric mice with germline transmission were backcrossed onto a C57Bl/6 (Ly-5.2) background. For the cell sorting and five-fragment embryo dissection experiments, Swiss Webster females were mated with male mice heterozygous for the GFP knock-in GFP-Bry^{+/−}. For the cell mixing experiments, transgenic male mice homozygous for the neomycin or hygromycin genes (Taconic Farms) were mated with Swiss Webster females. Embryos were staged according to morphological criteria²⁷.

Embryos were removed from decidua and Reichert's membrane, and dissected in IMDM containing 0.2% BSA (stock 1% BSA in 1 × PBS buffer). The dissection of GFP-Bry^{+/−} embryos into fragments was performed under a Leica MZFLIII fluorescence dissecting stereomicroscope using tungsten needles (Fine Science Tools). Embryos and embryo fragments were dispersed into a single cell suspension by incubation in a 2.5% trypsin-EDTA solution (Sigma) for 4 min at 37 °C and by pipetting up and down.

Haemangioblast and haematopoietic progenitor assays

The haemangioblast assay used was a modification of the blast assay developed in the

ES/embryoid body system. Embryo cell suspensions were plated in 1% methycellulose containing 10% FCS (Summit), VEGF (5 ng ml⁻¹), insulin-like growth factor-1 (IGF-1; 50 ng ml⁻¹), leukaemia inhibitory factor (LIF; 1 ng ml⁻¹), interleukin-6 (IL-6; 5 ng ml⁻¹) and 25% D4T endothelial cell conditioned medium². Colonies were grown in either 10 mm dishes or 24-well or 96-well plates in low oxygen incubators (5% oxygen). Haemangioblast colonies were further expanded using previously published expansion conditions¹. Haematopoietic progenitors were assayed in 1% methycellulose containing 10% protein-free hybridoma medium (Gibco/BRL), 15% plasma-derived serum (Antech), c-kit ligand (KL; 1% conditioned medium), IL-3 (1% conditioned medium), granulocyte-macrophage colony-stimulating factor (GM-CSF; 3 ng ml⁻¹), IL-11 (5 ng ml⁻¹), erythropoietin (2 U ml⁻¹), IL-6 (5 ng ml⁻¹) and thrombopoietin (1% conditioned medium). Primitive erythroid colonies were counted from day 4–5 whereas definitive macrophage, erythroid-macrophage and mast colonies were counted after 7–10 days of culture.

Gene expression analysis

Expression analyses of haemangioblast/blast colonies, the non-adherent and adherent cell populations from expanded haemangioblast colonies, and sorted cell populations were performed using a modified global cDNA amplification protocol^{2,28}.

Total RNA from pooled fragments of dissected neural plates stage embryos was harvested using the Absolutely RNA Microprep kit (Stratagene) and reverse transcribed with Omniscript RT (Qiagen) to generate cDNA. Primers used for PCR amplification of *brachyury*, *Flk-1* and β -actin were as previously reported¹⁷. The following additional primers were also used: *Gata1* (forward) 5'-CATGGCCCTTGTGAGGCCAGAGA-3', *Gata1* (reverse) 5'-ACCTGATGGAGCTTGAATAAGAGGC-3'; *Cer1* (forward) 5'-CCAGGCTTGAAGATCTGGAAGA-3', *Cer1* (reverse) 5'-GTCTTACCACATGCACTGACA CTCT-3'; *Mesp1* (forward) 5'-GTTCTGTACGCAGAAACAGCATC-3', *Mesp1* (reverse) 5'-CAAGGAGGGTGTGAATGGTACAGT-3'.

Neomycin and hygromycin gene detection

PCR reactions for the neomycin and hygromycin genes were performed using the Advantage 2 PCR system (BD Biosciences Clontech) on genomic DNA isolated from the non-adherent and adherent cell populations of the liquid-expanded haemangioblast colonies. The primers used were as previously published².

Fluorescence-activated cell sorting

Embryo cells at a concentration of 1×10^6 ml⁻¹ were incubated with a biotinylated anti-Flk-1 antibody followed by incubation with streptavidin-PE-Cy5 (BD Pharmingen) and sorted on a MoFlo high-speed cell sorter (Cytomation). For the limiting dilution analysis (Fig. 3f), GFP⁻Flk-1⁺, GFP⁺Flk-1⁺ and GFP⁺Flk-1⁻ cells were sorted directly into haemangioblast conditions in 96-well plates. Out of three experiments, one was performed using duplicate wells containing 3,300, 5,700 or 10,000 cells each, and two experiments were performed using only one well for each condition.

Immunofluorescence for CD31 and SMA expression

Haemangioblast colonies were cultured on fibronectin-coated glass coverslips in IMDM medium containing VEGF (5 ng ml⁻¹) and basic fibroblast growth factor (bFGF; 10 ng ml⁻¹) for 5–8 days. The coverslips were fixed with 4% paraformaldehyde for 15 min at room temperature, incubated with biotinylated anti-mouse CD31 (BD Pharmingen) and anti-mouse SMA (NeoMarkers) in 1 × PBS buffer for 1 h, washed five times (10 min washes) and incubated with streptavidin-Cy3 (Sigma) and anti-mouse-FITC (Biosource International) for 1 h. After five washes the coverslips were inverted onto a drop of 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc.) on slides and viewed under an inverted fluorescence microscope.

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The role of *barren stalk1* in the architecture of maize

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The architecture of higher plants is established through the activity of lateral meristems—small groups of stem cells formed during vegetative and reproductive development. Lateral meristems generate branches and inflorescence structures, which define the overall form of a plant^{1–3}, and are largely responsible for the evolution of different plant architectures³. Here, we report the isolation of the *barren stalk1* gene, which encodes a non-canonical basic helix-loop-helix protein required for the

initiation of all aerial lateral meristems in maize. *barren stalk1* represents one of the earliest genes involved in the patterning of maize inflorescences, and, together with the *teosinte branched1* gene⁴, it regulates vegetative lateral meristem development. The architecture of maize has been a major target of selection for early agriculturalists and modern farmers, because it influences harvesting, breeding strategies and mechanization. By sampling nucleotide diversity in the *barren stalk1* region, we show that two haplotypes entered the maize gene pool from its wild progenitor, *teosinte*, and that only one was incorporated throughout modern inbreds, suggesting that *barren stalk1* was selected for agronomic purposes.

barren stalk1 (*ba1*) is a spontaneous recessive mutation identified in 1928 (*ba1-ref*) from material collected by R. A. Emerson in South America⁵. Homozygous *ba1* mutant plants are unable to produce vegetative branches (tillers), female inflorescences (ears) and a normal apical male inflorescence, the tassel⁶. The tassel of *ba1* mutants is unbranched, shortened and predominantly sterile owing to the often complete lack of spikelets, the short branches

bearing florets that represent the basic unit of grass inflorescences (Fig. 1a–d, l). Double mutant analysis with *teosinte branched1* (*tb1*), an enhanced tillering mutant, and *in situ* hybridization on immature tassels with *knotted1*, a marker for meristematic cells, showed that lateral meristems in the aerial portion of *ba1* mutant plants fail to initiate during both vegetative and reproductive development, whereas the shoot apical meristem and inflorescence meristem, which define the main axis of aerial growth, develop normally⁶.

We isolated the *ba1* gene through a candidate gene approach, using the recently isolated *LAX* gene of rice² to probe an immature tassel complementary DNA library and a bacterial artificial chromosome (BAC) library. The *lax* mutant fails to initiate lateral meristems during the reproductive phase, resulting in an almost completely unbranched inflorescence with few or no spikelets. Vegetative lateral meristems, however, are unaffected². Even though *ba1*, unlike *lax*, affects both vegetative and reproductive development, the map positions of *ba1* and *lax* are in syntenic regions of chromosomes 3L and 1L, respectively, suggesting that both mutants

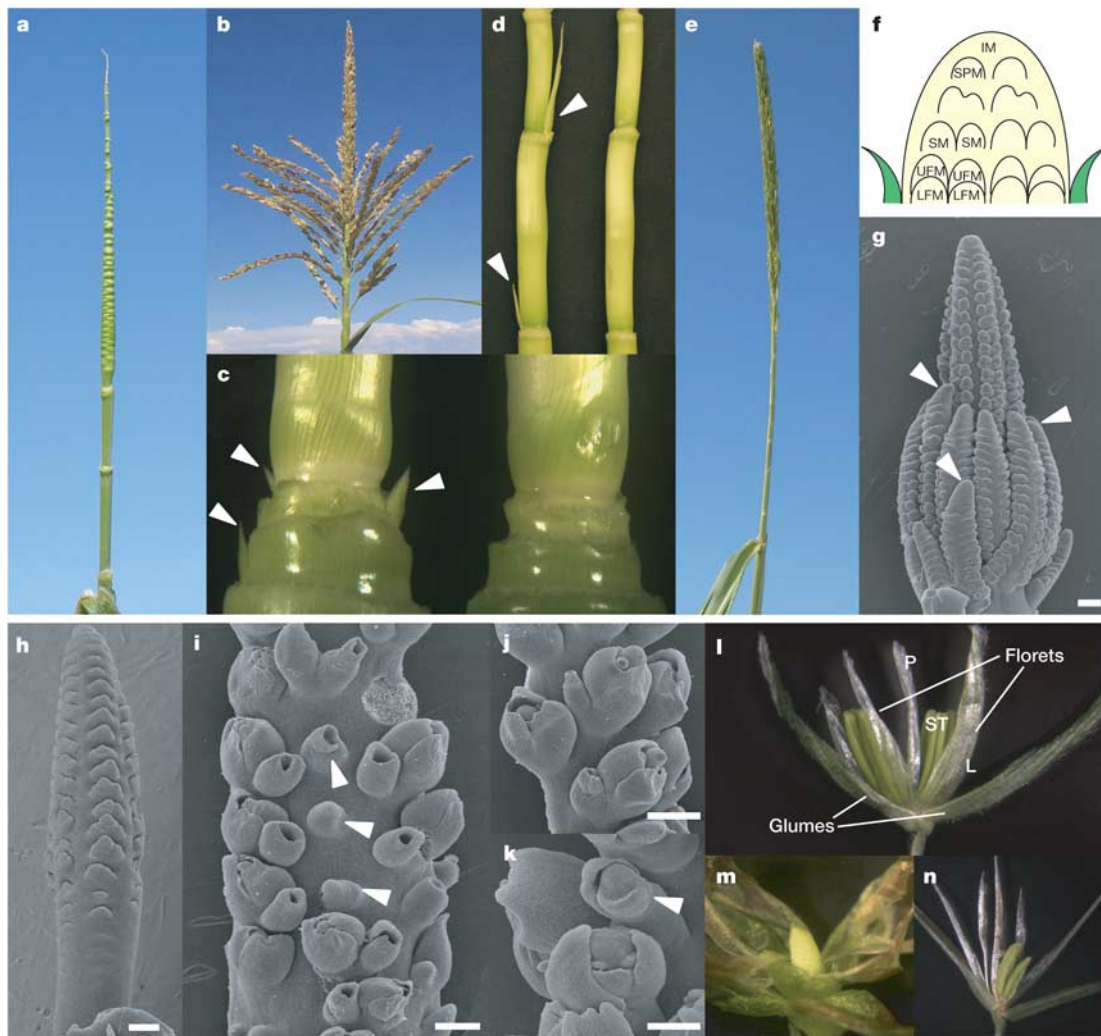


Figure 1 Effects of *ba1* mutations on maize development. **a**, *ba1-ref* tassel. **b**, Wild-type tassel. **c**, **d**, Axillary buds during vegetative (**c**) and reproductive (**d**) development in wild-type (left) and *ba1-ref* mutant plants (right). Upon removal of leaves, axillary buds (arrowheads) are only visible in wild-type plants. **e**, *ba1-mum1* mutant tassel. Spikelets are borne on the central rachis. **f**, Schematic representation of the acropetal development of maize inflorescences and of the lateral meristems involved. IM, inflorescence meristem; SPM, spikelet-pair meristem; SM, spikelet meristem; UFM/LFM, upper/lower floral meristem. **g–k**, SEM of immature tassels. **g**, Wild type showing branch meristems

(arrowheads). **h**, *ba1-mum1*. **i**, *ba1-mum1* (later stage); several meristems are arrested in development (arrowheads). **j**, *ba1-mum1* spikelet pairs. **k**, *ba1-mum3* spikelets; white arrowhead points to an undeveloped spikelet meristem. **l**, Wild-type tassel spikelet. Each floret is composed of lemma (L), palea (P) and three stamens (ST). **m**, *ba1-mum1* mature spikelet. A sterile structure is produced in place of reproductive organs. **n**, *ba1-mum3* spikelet. The upper floret is missing; the lower floret has a reduced number of stamens. Scale bars, 200 μ m.

could carry defects in orthologous genes. Library screens led us to identify a single gene. We subsequently cloned its corresponding genomic region in the *ba1-ref* mutant and identified a 6.5-kilobase (kb) insertion in the proximal regulatory region at -306 base pairs (bp) from the predicted start codon (Fig. 2a). This insertion is a *Helitron*; that is, a recently discovered class of transposable elements⁷. As the *ba1-ref* allele is the only known allele in mutant collections, we searched for mutations in the candidate gene by screening a *Mutator*-tagged population (Trait Utility System Corn, Pioneer Hi-Bred International) and by sequencing the gene from a *ba1*-like mutant found to be segregating in an ethylmethanesulphonate-induced M2 family. The identification of three insertions and a point mutation in the same gene, each showing a *ba1*-like tassel phenotype (Fig. 1a, e) and suppression of ear and tiller formation, confirmed that we had isolated the *ba1* gene. These alleles were named *ba1-mum1*, *ba1-mum2*, *ba1-mum3* and *ba1-IL*, respectively (Fig. 2a).

The *ba1* gene is intron-less, as is *LAX*², and the cDNA sequence presents an open reading frame (ORF) of 660 bp. The comparison between BA1 and LAX putative amino acid sequences reveals an overall identity of 62% (Fig. 2b). The *ba1* gene is predicted to encode a small protein of 219 amino acids with a basic helix-loop-helix (bHLH) domain, which is the defining feature of the bHLH family of transcription factors. This domain is 100% conserved when compared to LAX (Fig. 2b,c). The bHLH domain consists of an amino-terminal basic region involved in DNA binding, and a carboxy-terminal HLH region, which leads to the formation of homo- or heterodimers^{8,9}. bHLH transcription factors are important components of eukaryotic transcription networks in many biological pathways^{8,9}. Although this family of transcription factors represents the second largest in the *Arabidopsis* genome, only a few members involved in developmental processes have been characterized in detail^{9,10}. The predicted BA1 bHLH domain lacks the glutamic acid at position 9 in the basic region that was demon-

strated in non-plant bHLH proteins to be necessary for DNA binding to the canonical E-box site (Fig. 2c)⁸⁻¹⁰. Although no significant similarities are observed outside of the bHLH domain, BA1 as well as the recently characterized INDEHISCENT (IND) protein in *Arabidopsis*¹⁰ belong to a subgroup of proteins that share an atypical bHLH domain that is suggested to bind DNA through recognition of a different motif or together with a partner protein⁸⁻¹⁰ (Fig. 2c).

The earliest events in tassel development^{11,12} include the formation on the flanks of the main inflorescence meristem of a few branch meristems that develop into the long branches of mature tassels, and of several rows of spikelet-pair meristems along the sides of the branches and the central spike. Spikelet-pair meristems give rise to two spikelet meristems, each of which initiates two floral meristems: the upper and lower floral meristems (Fig. 1f,g). This specific sequence of branching events by intermediate lateral meristems eventually leads to the formation of two paired spikelets (pedicellate and sessile spikelets), each consisting of a pair of glumes (modified leaves) enclosing a short branch terminating in an upper and a lower floret (Fig. 1l) composed of lemma, palea, lodicules and stamens¹¹⁻¹³.

Homozygous *ba1-mum1* and *ba1-mum3* plants manifest a weaker phenotype than the *ba1-ref* mutant, typically having a central spike of normal length with spikelets forming along the upper portion, but still lacking basal branches (Fig. 1e). *ba1-mum2* and *ba1-IL* tassels are, however, as severely affected in branching and spikelet formation as seen in the *ba1-ref* tassels. The spikelets that form in the upper portion of *ba1-mum1* and *ba1-mum3* tassels manifest a wide range of defects. Scanning electron microscopy (SEM) on immature *ba1-mum1* and *ba1-mum3* tassels reveals that the development of all lateral meristems is affected. No branch meristems are formed, and several spikelet-pair meristems remain undeveloped, originate single spikelets or divide abnormally (Fig. 1h-k). Some spikelet meristems appear smaller and produce tubular structures in place of glumes (Fig. 1i-k). Once matured, mutant spikelets are either empty or bear a varying number of floral organs in just one of the two florets or in both florets (Fig. 1m, n). These defects indicate that a functional *ba1* gene is required early in inflorescence development for branch meristem and spikelet-pair meristem initiation, and later for the correct establishment of all subsequent lateral meristems, either having a role in their maintenance or possibly regulating organ formation during spikelet and floret development. As meristem size correlates with the number of organs formed¹⁴, we favour the view that *ba1* is necessary for establishing the appropriate population of meristematic cells, which is in turn required for the correct partitioning of these cells during all branching events, as already suggested for *barren inflorescence2*, a mutant similarly impaired in lateral meristem formation¹⁵.

Expression analysis via *in situ* hybridizations during vegetative and reproductive development supports these deduced functions of BA1 (Fig. 3). Sections of young seedlings show a defined signal on the adaxial (facing towards the stem) side of initiating axillary meristems, whereas no expression is observed in the shoot apical meristem (Fig. 3a). After the transition to the reproductive phase, *ba1* is first detected in a narrow arc of cells immediately above the region where branch meristems and spikelet-pair meristems will initiate (Fig. 3b, c). As these branch meristems and spikelet-pair meristems become visible, the expression persists in a position adjacent to the initiating meristems (Fig. 3b, c) and is later localized in the dividing spikelet-pair meristems, in the region between the two developing spikelet meristems (Fig. 3d). During floral meristem formation, we detected the transcript in a region of cells between the upper floral meristem and lower floral meristem, and in a more diffuse pattern in the upper floral meristem (Fig. 3e). As spikelets develop, *ba1* expression remains localized to the sides and to the base of both spikelets (Fig. 3f). In sections through ear spikelets,

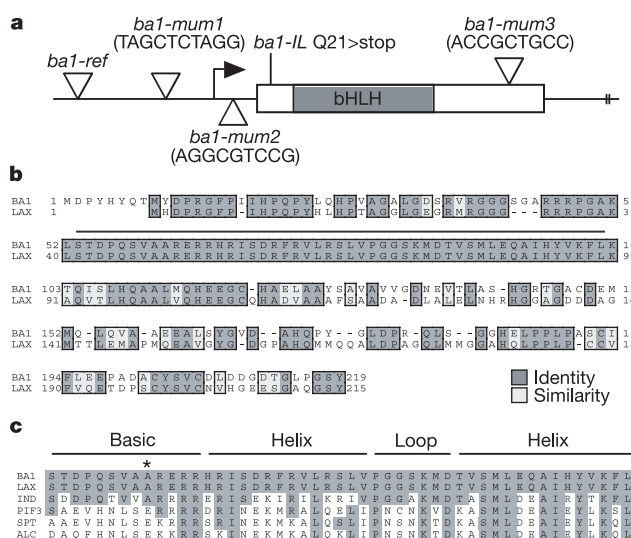


Figure 2 *barren stalk1* encodes a non-canonical bHLH protein. **a**, *ba1* gene schematic showing the location of mutations in characterized alleles. The rectangle represents the coding region with the bHLH domain shown in grey. The arrow and vertical bars represent the transcription start and end, respectively. In parenthesis are the sequences of the target site duplication resulting from *Mutator* insertions. **b**, Comparison of BA1 and LAX predicted amino acid sequences. The bHLH domain is indicated by an overhead horizontal line. **c**, Sequence alignment of BA1 bHLH domain with several related proteins characterized in plants: LAX, IND, PHYTOCHROME INTERACTING FACTOR3 (PIF3), SPATULA (SPT) and ALCATRAZ (ALC)^{2,9,10}. The asterisk marks the alanine residue at position 9 of BA1, LAX and IND basic domain.

when the reproductive organs are initiating, *ba1* expression is observed on the adaxial side of the lower floret but not in developing floral organ primordia (Fig. 3g). A comparatively faint signal is present in developing anthers of young tassel spikelets (Fig. 3h). No expression is seen in sections of *ba1-ref* tassels, but a shortened transcript is weakly detectable through northern hybridizations (data not shown), suggesting that the smaller transcript is unstable.

In every branching event, from the formation of branch meristems to the formation of floral meristems, *ba1* retains a clear and specific expression pattern, localized in the regions where this branching occurs. These observations, together with the mutant phenotypes, suggest that a conserved mechanism in maize regulates the formation of lateral meristems during both vegetative and reproductive development. The reiterative adaxialized expression domain observed during the initiation of all lateral meristems indicates that these *ba1*-expressing cells have a specific identity and might be involved in transmitting an endogenous signal to regulate downstream genes controlling lateral meristem initiation and outgrowth. Several mutants affected in lateral meristem formation have been related to failures in polar auxin transport or signalling. Polarized gradients of auxin were recently proposed as a common module for several developmental processes^{16,17}, raising the possibility that *ba1* could be involved in an auxin signalling pathway.

The morphology of modern maize is derived from that of its wild progenitor teosinte through a domestication process that is still poorly understood^{4,18–20}. A quantitative trait locus (QTL) analysis of

maize and teosinte identified five major chromosomal regions that explain most of their morphological differences^{4,19}, essentially accounting for lateral branch development and for the positioning and morphology of female inflorescences^{4,19,21}. So far, only the *teosinte branched1* gene, a TCP transcription factor, has been clearly implicated in the domestication of maize architecture by its identity with the QTL on the long arm of chromosome 1 (refs 4, 19–22). Genetic evidence supports the existence of an epistatic interaction between another major QTL on the long arm of chromosome 3 (QTL 3L) and *tb1* (refs 19, 23). QTL 3L was proposed as an upstream regulator of *tb1* (ref. 23). TB1 is a repressor of lateral meristem outgrowth and the *teosinte branched1* mutant is characterized by a severe loss of apical dominance, which results in an unrestrained outgrowth of axillary buds along the main stem⁴. Accordingly, expression of *tb1* is first detected during the formation of vegetative axillary meristems²¹. *barren stalk1* maps to the genomic region encompassing QTL 3L, and the *ba1* mutation is epistatic to *tb1*, suppressing tiller formation in double mutant plants, suggesting that *ba1* acts upstream of *tb1* in the pathway of vegetative lateral meristem formation⁶. In order to clarify the relationship between these two genes, we quantitatively analysed *tb1* expression in *ba1* mutant shoots and developing tassels, and *ba1* expression in *tb1* mutant seedlings (Fig. 4a). The reduction in *tb1* transcript levels in both *ba1-ref* seedling and tassel samples confirms that *ba1* activity is required for normal levels of *tb1* expression. Furthermore, the normal level of expression of *ba1* in *tb1* mutant seedlings, together with the *ba1* expression pattern adjacent to initiating meristems, suggest that *ba1* is required for axillary meristem formation but not for axillary shoot elongation.

We then investigated whether selection acted on *ba1* during maize domestication or improvement. One expectation for a selected gene is a change in the pattern of nucleotide diversity, as the favourable allele(s) is brought to higher frequency by human selection^{20,22,24}. We analysed sequence diversity at *ba1* in 14 diverse maize inbreds, 16 diverse maize landraces, and 14–17 teosinte samples (*Zea mays parviglumis* and *Z. mays mexicana*) for three distinct regions of *ba1* (Fig. 4b, c). Two standard selection tests (Tajima's D^{25} and the Hudson–Kreitman–Aguadé (HKA) test²⁶) were consistent with neutral evolution at *ba1* in maize landraces, but the HKA test showed a highly significant deviation from neutrality for inbreds ($P < 0.0002$). Moreover, for *ba1*, maize inbreds possess only 3% of the diversity found in teosinte (Fig. 4b), a value that is much smaller than the 30% figure for starch pathway genes²⁷, which were targets of human selection, and nearly as small as that for the domestication gene *tb1* (ref. 22). The low diversity among inbreds is a function of their possessing a single haplotype, as shown by a survey of 86 diverse maize inbreds (Supplementary Data). Thus, although the standard tests provide no evidence that *ba1* was under selection during domestication, they suggest that it was under selection during maize improvement, reducing modern maize inbreds to a single basic haplotype (haplotype I, see below).

Although the standard tests of selection during domestication for *ba1* were not significant, other aspects of the sequence data are atypical of genes that passed through the domestication process in a neutral fashion. First, maize landraces possess only two basic haplotypes (I and II; Fig. 4c) in contrast to the multiple haplotypes typically observed at neutral loci in maize²⁴. Haplotype I is found in maize from throughout the Americas, but haplotype II is found only in the central Mexican highlands. Second, these two maize haplotypes occur with a patterned distribution in teosinte. The populations of the *parviglumis* subspecies from the region of maize domestication (southwestern Mexican lowlands²⁰) possess haplotype I plus multiple other haplotypes but not haplotype II. In contrast, both haplotypes I and II occur in the *mexicana* subspecies from the central Mexican highlands. Third, in maize there is no evidence of recombination between haplotypes I and II, and accordingly linkage disequilibrium (LD) at *ba1* in maize is

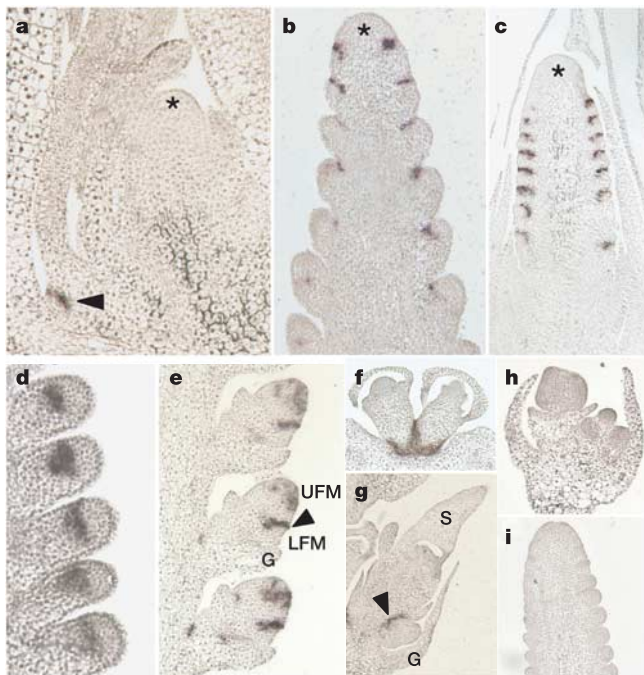


Figure 3 *barren stalk1* expression by *in situ* hybridization. **a**, Longitudinal section of a 5-day-old seedling showing *ba1* expression on the adaxial side of the initiating vegetative axillary meristem (arrowhead). Asterisk, shoot apical meristem. **b**, **c**, Longitudinal sections of immature tassel (**b**) and ear (**c**). Asterisk marks the inflorescence meristem. **d**, Longitudinal oblique section of a series of dividing spikelet-pair meristems. **e**, Longitudinal section of spikelets in which upper floral meristems and lower floral meristems are forming. Signal is detected in the upper floral meristem and in the region demarcating the upper and lower floral meristems (arrowhead). G, glume primordia. **f**, Cross-section of an ear spikelet pair. **g**, Longitudinal section of ear spikelets. Strong expression is observed adaxial to the lower floret (arrowhead). S, developing silk. **h**, Tassel spikelet. **i**, Ear, sense control.

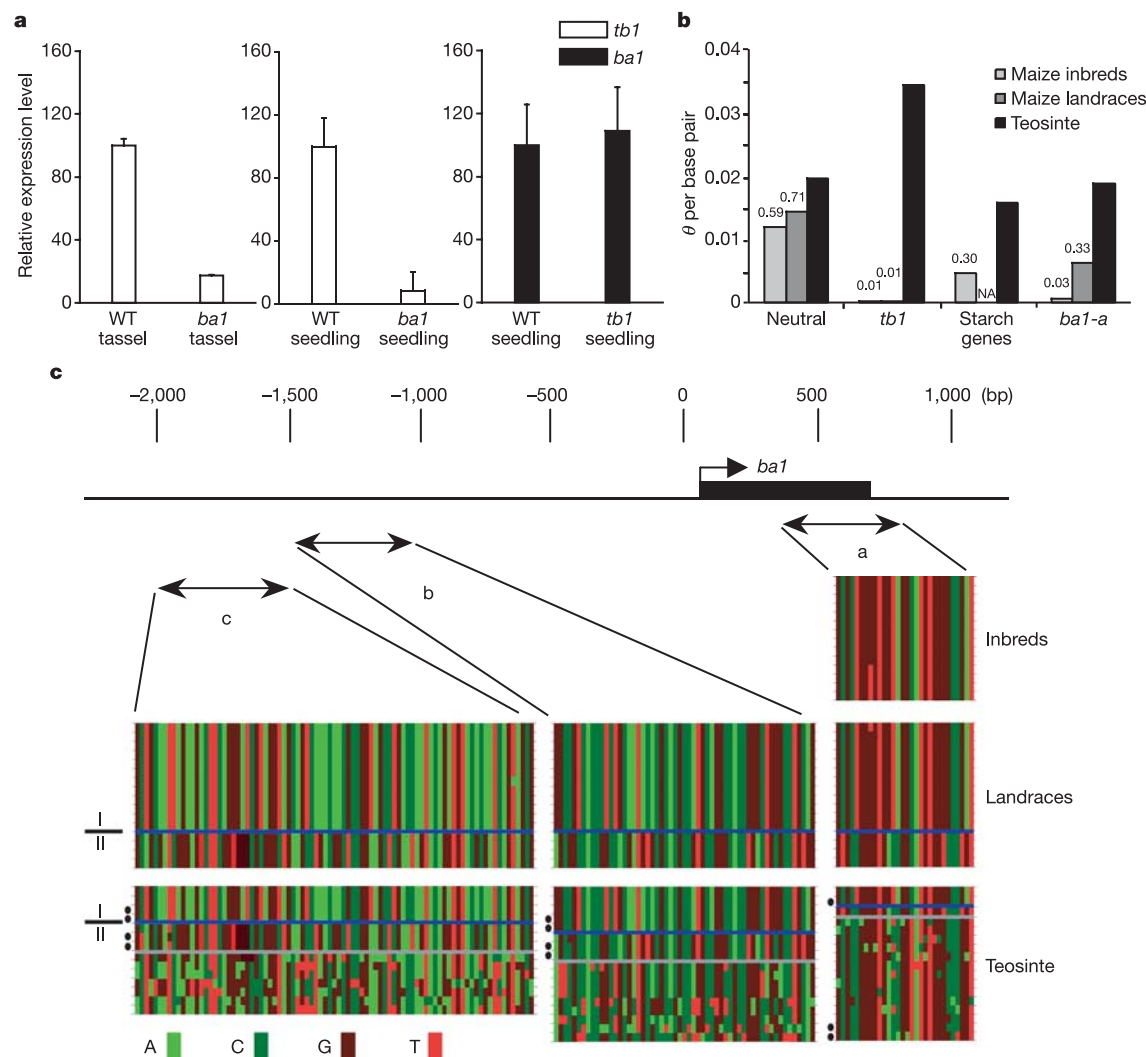


Figure 4 *barren stalk1* as a candidate for QTL 3L. **a**, Expression analysis of *tb1* and *ba1* in *ba1* and *tb1* mutants, respectively, by RT-quantitative PCR. Values for the y axis are arbitrary units of expression level relative to actin. Error bars indicate standard deviation of at least four replicates. WT, wild type. **b**, Comparison of silent nucleotide polymorphism (θ) between maize and teosinte in the coding region of the *ba1* locus (*ba1-a*). The numbers above the bars indicate the fold reduction in diversity between maize and teosinte. Values for neutral genes (*csu1171*, *asg11*, *csu1132*, *bz2*, *glb1*, *adh1* and *csu381*)²⁴, maize

starch pathway genes (*ae1*, *bt2* and *su1*)²⁷ and the domestication gene *tb1* are indicated. **c**, Single nucleotide polymorphism survey between maize and teosinte. Three different regions of the *ba1* locus (a, b, c) were analysed. Each array consists of sequenced samples (rows) and polymorphic sites (columns). Haplotypes I and II are separated by the blue lines; other teosinte haplotypes appear below the grey lines. Dots mark the *mexicana* subspecies sequences.

very high ($r^2 = 0.96 - 1.0$) whereas in teosinte it is modest ($r^2 = 0.06 - 0.31$).

Although uncertainty remains about the role of *ba1* during the domestication process, these observations can be explained as follows. Initially, *ba1* was a target of selection during maize domestication from the *parviglumis* subspecies in the Mexican lowlands and as a consequence only haplotype I entered the maize gene pool. Later after maize spread to the highlands, maize acquired haplotype II from the *mexicana* subspecies via introgression. Thus, haplotype II is found in sympatric populations of maize and the *mexicana* subspecies at high elevations in central Mexico. The high LD observed for *ba1* in maize is a consequence of the admixture with the *mexicana* subspecies. Finally, because of the admixture with the *mexicana* subspecies, the standard tests of selection are nonsignificant for maize landraces, despite the fact that *ba1* was under selection during the domestication process. If this model is correct, then future analyses should show that both haplotypes I and II are associated with a more maize-like phenotype as compared

with the multiple other haplotypes found only in teosinte.

The evidence suggests that *ba1* was under selection during maize improvement and encourages further investigation to determine whether *ba1* corresponds to the QTL 3L implicated in maize domestication^{19,23}. Mutations in *ba1* produce a plant manifesting strong apical dominance, whereas mutations in *tb1* cause the opposite effect, namely a severe loss of apical dominance. A balance between the activities of these two genes may have been an important contributor to the domestication and improvement of maize. □

Methods

Gene cloning

A full-length LAX cDNA clone was used to screen at medium stringency ($\sim 57^\circ\text{C}$) a cDNA library from RNA of immature tassels (1–3 mm), cloned in HybriZAP-2.1 vector (Stratagene). Hybridizing clones were recovered according to manufacturer's instructions (Stratagene). The same probe was used to isolate a 4.5-kb fragment of the genomic region harbouring the *ba1* gene by screening a BAC library (NSF B73, Clemson University Genomic Institute). The mutant *ba1-ref* locus was characterized as follows. The coding sequence was amplified by polymerase chain reaction (PCR). Southern analysis on

genomic DNA (*Bam*HI digest) revealed an insertion in the proximal promoter region, whose sequence and location were subsequently determined. A proximal 1.5-kb *Bam*HI fragment was isolated through iPCR. The distal *Bam*HI fragment was cloned using the ZAP Express predigested vector kit (Stratagene), following the manufacturer's instructions. The whole insertion was eventually obtained using the Elongase amplification system (Invitrogen).

New *ba1* mutant alleles were identified at Pioneer Hi-Bred International, as previously described²⁸, via PCR (Supplementary Methods). New material is available for non-commercial research purposes upon acceptance and signing of an appropriate material transfer agreement. The *ba1-IL* (03IL-A619TR-996) point mutation was identified by sequencing the *ba1* gene from mutant plants segregating in an ethylmethane-sulphonate-generated M2 family (<http://www.maizegdb.org/mip>). We used ClustalW (MacVector 6.5 [K]) for sequence comparisons.

Expression analysis and SEM

Tissue samples were fixed for both *in situ* sectioning and SEM as previously described¹³. SEM specimens were viewed on a Quanta 600 microscope. *In situ* hybridizations were performed as previously reported²⁹. Antisense probes for *ba1* were synthesized with T7 polymerase using DIG RNA labelling mixture (Roche). Both *Hind*III and *Pst*I 3' fragments of the *ba1* cDNA (~500 and 300 bp, respectively) were used for *in situ* hybridizations. *ba1* sense probe template was amplified from the same *Hind*III fragment using a chimeric T7 promoter primer.

Total RNA from pooled seedlings (20 days old) and immature tassels (1.5–2.5 cm) was extracted using standard procedures. RNA samples were further purified (Qiagen RNeasy mini kit) and 2–5 µg were treated with DNase and subsequently reverse-transcribed using the Superscript first-strand synthesis system for RT–PCR, following the manufacturer's instructions (Invitrogen). RT–PCR reactions were run in a LightCycler (Roche) using Hot Start SYBR green reaction mix (Roche) and analysed using the LightCycler relative quantification software (version 1.0, Roche). Samples for expression level comparison are in the same genetic background. Primer sequences are available on request.

Nucleotide diversity survey and statistical analysis

Nucleotide diversity in the *ba1* locus was determined for three fragments (regions a, b and c) from 16 maize landraces previously described²⁴, 14 inbreds and 14–17 teosinte individuals (Supplementary Methods and Supplementary Table 1). Nucleotide polymorphism (θ), linkage disequilibrium (r^2) and Tajima's *D* statistic were calculated using DnaSP version 4.0 (ref. 30). The HKA test was performed using *Tripsacum* as the preferred outgroup for the divergence estimate, and *Zea diploperennis* in cases where the *Tripsacum* sequence could not be obtained. PCR products for *Z. diploperennis* and *Tripsacum brachyotum*, potentially heterozygous for *ba1*, were cloned using the PCR 2.1-TOPO kit (Invitrogen) and at least four clones were sequenced. A joint HKA test across the six neutral loci was obtained by summing the individual χ^2 values.

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Correspondence and requests for materials should be addressed to R.J.S. (rschmidt@ucsd.edu). Sequences are deposited in GenBank under accession numbers AY683001, AY683002 and AY645947. Other accession numbers are listed in Supplementary Table 1.

The *BCL6* proto-oncogene suppresses p53 expression in germinal-centre B cells

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The human proto-oncogene *BCL6* encodes a BTB/POZ-zinc-finger transcriptional repressor that is necessary for germinal-centre formation and is implicated in the pathogenesis of B-cell lymphoma^{1–3}. The precise function of *BCL6* in germinal-centre development and lymphomagenesis is unclear because very few direct *BCL6* target genes have been identified^{4–7}. Here we report that *BCL6* suppresses the expression of the *p53* (also known as *TP53*) tumour suppressor gene and modulates DNA damage-induced apoptotic responses in germinal-centre B cells. *BCL6* represses *p53* transcription by binding two specific DNA sites within the *p53* promoter region and, accordingly, *p53* expression is absent in germinal-centre B cells where *BCL6* is highly expressed. Suppression of *BCL6* expression via specific short interfering RNA leads to increased levels of *p53* messenger RNA and protein both under basal conditions and in response to DNA damage. Most notably, constitutive expression of *BCL6* protects B cell lines from apoptosis induced by DNA damage. These results suggest that an important function of *BCL6* is to allow germinal-centre B cells to tolerate the physiological DNA breaks required for immunoglobulin class switch recombination and somatic hypermutation without inducing a *p53*-dependent apoptotic response. These findings also imply that deregulated *BCL6* expression contributes to lymphomagenesis in part by functional inactivation of *p53*.

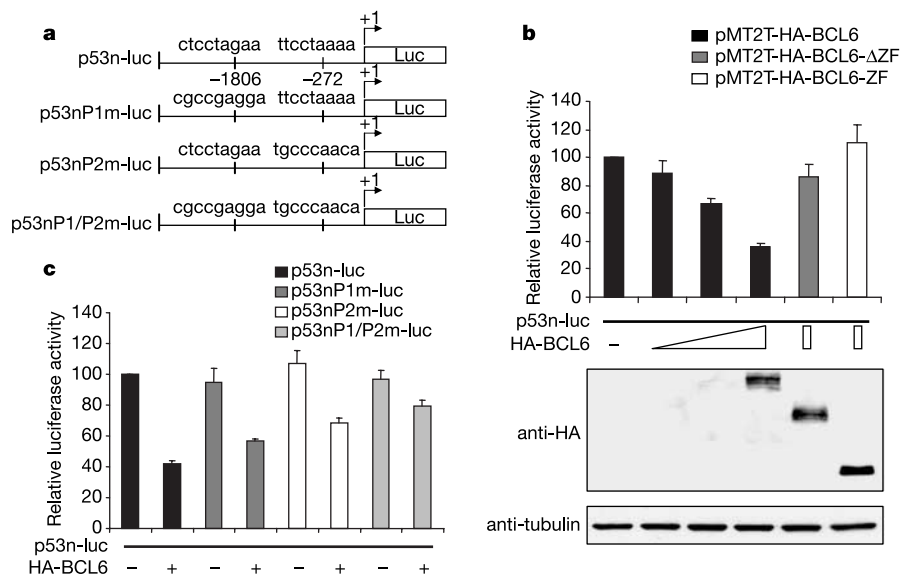


Figure 1 Transcriptional repression of the p53 promoter by BCL6. **a**, Schematic representation of the p53 promoter-driven luciferase reporter construct (p53n) and its derivatives. **b**, Vectors expressing BCL6 or mutants (BCL6-ΔZF and BCL6-ZF) and p53n-luc were transiently co-transfected into 293T cells and luciferase activity was measured 48 h post-transfection. Each experiment was done in duplicate and data

represent the mean $\pm$ s.d. of three independent experiments. The lower panel shows the expression levels of BCL6 proteins by western blotting. **c**, The p53n-luc and three derivatives were transiently transfected with or without vector expressing BCL6 into 293T cells and luciferase activity was compared 48 h post-transfection.

Within the B-cell lineage, the BCL6 protein is expressed at high levels only in mature B cells within the germinal centre⁸, the structure where immunoglobulin genes undergo somatic hypermutation and class switch recombination (CSR)^{9,10}. BCL6 is necessary for germinal-centre development and for T-cell-dependent humoral immune responses because mice lacking BCL6 do not form a germinal centre and lack antibody affinity maturation^{2,3}. In germinal-centre-derived B-cell lymphomas, such as follicular lymphoma and diffuse large B-cell lymphoma (DLBCL), the BCL6 gene is commonly involved in chromosomal translocations that deregulate its expression by a mechanism called promoter substitution¹¹. To understand the precise function of BCL6 it is critical to identify the genes whose expression is modulated by BCL6 via direct binding to their promoter regions. Although a number of potential targets has been identified by examining genes that are differentially expressed upon BCL6 induction, these may include a large number of indirect targets⁴. In fact, functional and biochemical evidence of direct transcriptional repression by BCL6 is available for only a few genes⁵⁻⁷.

In the course of studies aimed at identifying candidate BCL6 direct target genes, we unexpectedly uncovered several known transcriptional targets of the p53 tumour suppressor, including the cell-cycle arrest gene *p21* (also known as *CDKN1A*) and the putative anti-apoptotic gene *PIG7* (also known as *LITAF*) (refs 12, 13). However, we failed to obtain any evidence demonstrating that BCL6 can directly regulate these p53 responsive genes, consistent with the observation that the promoter regions of these genes do not contain BCL6 binding sites. These observations prompted us to investigate whether BCL6 may downregulate p53 target genes via direct repression of p53 transcription, a plausible hypothesis given that p53 mRNA and protein expression are not detectable in germinal-centre B cells expressing BCL6 (see Supplementary Information Fig. S1) despite the fact that these cells are thought to be undergoing extensive genetic stress¹⁴⁻¹⁶.

We first searched the promoter region of the *p53* gene and found two closely located sequences that share an extensive homology with the preferred BCL6 binding sequence¹⁷. We then investigated whether BCL6 could repress the transcription of a reporter gene driven by the native p53 promoter region containing the two

putative BCL6 binding sites (Fig. 1a) in transient transfection/reporter assays. As shown in Fig. 1b, co-transfection of increasing amounts of a vector encoding BCL6 leads to a dose-dependent suppression of the p53 reporter expression that is comparable to

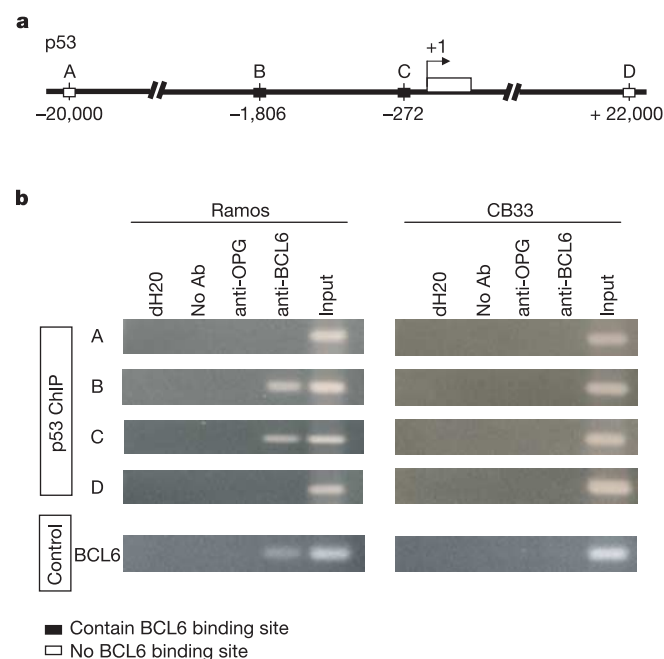


Figure 2 BCL6 binds to the p53 promoter region *in vivo*. **a**, Schematic representation of the human p53 promoter region. The four genomic fragments targeted for PCR amplification and their positions from the transcription initiation site are approximately indicated (B and C: target regions; A and D: control regions). **b**, ChIP assays were performed using Ramos cells and CB33 cells. PCR was performed in immunoprecipitated chromatin fragments using anti-BCL6 antibody or an irrelevant antibody (anti-OPG) as control. One sample was also processed with no antibody (Ab) to serve as a negative control. Input represents PCR amplification of total chromatin before immunoprecipitation. A known BCL6 binding sequence in the first exon of BCL6 gene itself was also amplified as a positive control for the ChIP assays (bottom panel).

that observed for other BCL6 promoter targets in transiently transfected 293T cells^{4-7,17}. Vectors that express BCL6 mutants lacking the amino-terminal transcriptional repressor domain (BCL6-ZF) or the carboxy-terminal DNA binding zinc-finger domain (BCL6-ΔZF) failed to suppress the expression of the *p53* reporter gene, indicating a bona fide DNA binding-dependent transcriptional effect by the wild-type BCL6. These results were further confirmed by the fact that mutation of each individual BCL6 binding site in the *p53* promoter region partially relieves transcriptional suppression, whereas mutation of both sites was associated with minor residual repression of the *p53* reporter gene (Fig. 1c). Collectively, these results indicate that BCL6 represses transcription driven by the *p53* promoter region and that this function requires the two BCL6 DNA binding sites to be present 5' to the *p53* transcription initiation site.

To determine the physiological relevance of these experimental findings, we investigated whether endogenous BCL6 binds the *p53* promoter region *in vivo* (Fig. 2a). Chromatin immunoprecipitation (ChIP) assays showed that the two DNA fragments (B and C) encompassing the BCL6 binding sites, but not two control fragments (A and D) are immunoprecipitated from BCL6-expressing B cells (Ramos cells) by anti-BCL6 antibodies, but not by species- and isotype-matched control antibodies (Fig. 2b, left panel). The specificity of the ChIP assays was further documented by the observation that fragments B and C are not immunoprecipitated from lymphoblastoid CB33 cells that do not express BCL6 (Fig. 2b, right panel). The ChIP assays were also internally controlled by the binding of BCL6 to a known BCL6-targeted sequence in the first

non-coding exon region of the *BCL6* gene itself⁶ (Fig. 2b, bottom panel). These results indicate that endogenous BCL6 polypeptides bind the *p53* promoter region *in vivo*.

To corroborate further the relationship between BCL6 and transcriptional suppression of *p53*, we examined whether inhibition of BCL6 expression would lead to de-repression of *p53* expression. To address this issue, B-cell clones with significantly reduced BCL6 expression were generated via retroviral transduction of short interfering RNA (siRNA) molecules specifically targeting BCL6 transcript (Fig. 3a). Because BCL6 may be required for B-cell survival, no clones that are completely devoid of BCL6 expression could be obtained. As shown in Fig. 3b, Ramos cells transduced with *BCL6* siRNA (lanes 3–4) significantly reduced endogenous BCL6 expression compared with the infected control cells (lanes 1–2). Analogous to most cell types, control-transduced clones express extremely low levels of *p53* mRNA (detectable only after 30 cycles of polymerase chain reaction (PCR)-mediated amplification) under basal conditions, because *p53* expression is induced by genotoxic stress¹⁸. Nonetheless, a reproducible ~2–3-fold increase in *p53* mRNA and protein expression is detectable in multiple clones transduced with the two different *BCL6* siRNA species (representative results shown in Fig. 3b, c). This small increase is significant because it is comparable to that observed upon stimulation with interferon (IFN)- α/β , a known inducer of *p53* gene transcription¹⁸, and it brings *p53* mRNA expression to levels comparable to those detectable in normal naïve B cells that lack BCL6 (see Fig. 3c).

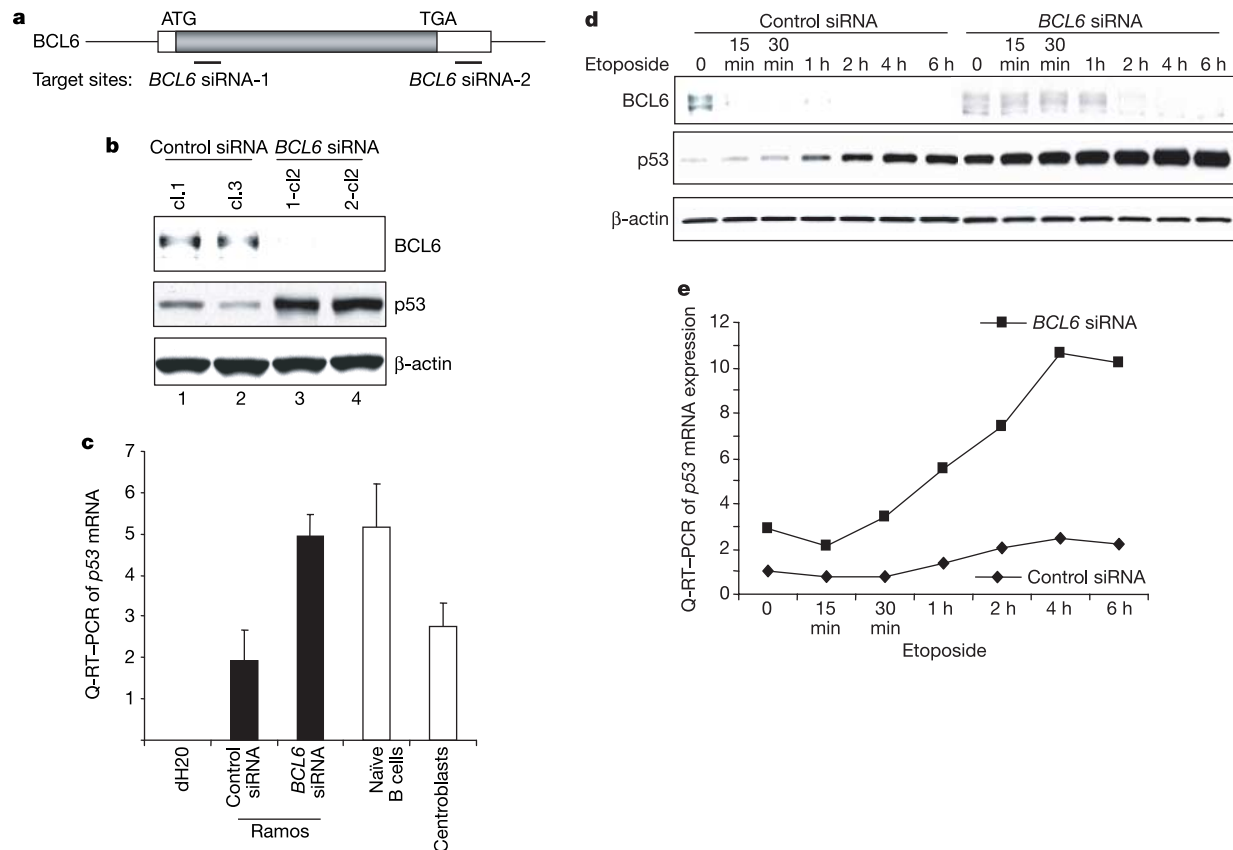


Figure 3 Suppression of BCL6 by siRNA induces p53 protein and mRNA expression. **a**, Schematic representation of two siRNA targeting sites in the BCL6 transcript. **b**, Western blot analysis of BCL6 and p53 protein expression in two control clones (cl.1 and cl.2) in lanes 1–2 and two BCL6 siRNA clones (1-cl2 and 2-cl2) targeting different regions of BCL6 transcript (lanes 3–4). **c**, Quantitative real-time RT-PCR (Q-RT-PCR) of

p53 mRNA in six different control clones and BCL6 siRNA clones (mean $\pm$ s.d.), naïve B cells and centroblasts isolated from three different sets of tonsillar mononuclear cells (mean $\pm$ s.d.). **d**, Western blot analysis of BCL6 and p53 protein expression in control and BCL6 siRNA cells that were treated with etoposide (20 μ M) for the indicated time. **e**, Quantitative real-time RT-PCR analysis of p53 mRNA levels from experiments in d.

These results suggest that p53 expression is under active repression by BCL6 under basal conditions in germinal-centre B cells.

Most notable was the effect of BCL6 ablation on p53 mRNA induction by genotoxic stress. Figure 3d, e shows that treatment of control Ramos clones with etoposide leads to the expected increase in p53 mRNA and protein expression as well as to downregulation of BCL6 expression by ubiquitin-mediated protein degradation¹⁹ (and our unpublished data); however, the levels of induction of p53 mRNA and protein are significantly increased (RNA, ~10-fold) in the clones expressing reduced BCL6 levels due to siRNA-mediated inhibition (Fig. 3d, e). Taken together, these results indicate that decreased BCL6 expression is directly associated with an increase in both the basal and inducible levels of p53, consistent with a role for BCL6 in suppressing p53 expression.

To examine the biological significance of these findings, we then investigated whether BCL6 expression could influence the cellular response to DNA damage via modulation of p53. To this end, we engineered Val B-cell lymphoma lines (which carry a normal p53 gene and display normal p53 responses) to constitutively express a mutant BCL6 protein haemagglutinin (HA)-BCL6 Δ PEST that can suppress transcription (not shown), but is resistant to etoposide-induced ubiquitin-mediated degradation (due to the absence of the critical PEST domain). Figure 4 shows that etoposide treatment of HA-BCL6 Δ PEST-transduced Val cells leads to downregulation of the endogenous wild-type BCL6 protein, but not of the exogenous HA-BCL6 Δ PEST protein (Fig. 4a). Consistent with BCL6-mediated suppression of p53, the induction of p53 and its target genes p21 and PUMA^{13,20} is significantly blunted in these cells, but not in the control-transduced cells (Fig. 4a). Most notably, the HA-BCL6 Δ PEST-transduced cells are significantly more resistant to etoposide-induced apoptosis, as documented by flow cytometric analysis using annexin V/7AAD (7-aminoactinomycin D) staining (Fig. 4b). Taken together, these results indicate that enforced BCL6 expression suppresses p53-mediated apoptotic responses to DNA damage in B cells.

The finding that BCL6 suppresses p53-mediated responses identifies a novel function for BCL6 in germinal-centre B cells. Within the B-cell lineage, BCL6 is expressed at high levels exclusively in B cells within the germinal centre⁸, the lymphoid structure in which B cells (centroblasts) proliferate at a very high rate⁹. Thus, suppression of p53 may prevent cell-cycle arrest and apoptosis facilitating the rapid expansion of the germinal centre. In addition, germinal-centre B cells undergo specific genome remodelling events, such as somatic hypermutation of multiple genes and CSR^{21,22}, which are thought to be mediated by DNA breaks^{14–16,23}. Therefore, BCL6-mediated inhibition of p53 expression may allow germinal-centre B cells to sustain the physiological genomic stress required for somatic hypermutation and CSR without inducing a p53-dependent apoptotic response. Our results are consistent with a model in which BCL6 prevents p53 responses to the low levels of DNA breaks associated with somatic hypermutation and CSR, whereas higher levels of DNA damage would downregulate BCL6 (Fig. 3d) and allow the resumption of a normal p53 response leading to exit from the germinal centre or apoptosis. Thus, mice lacking BCL6 expression may not be able to form a germinal centre^{2,3} because of an early p53 response preventing their proliferative expansion.

Approximately 45% of DLBCL, the most common human B-cell lymphoma, express BCL6 constitutively owing to chromosomal translocations or specific mutations that alter its promoter region^{24,25}. On the basis of the findings herein, these tumours can be considered functionally p53-negative, an observation supported by the findings that these tumours, but not DLBCL lacking BCL6 translocations, are virtually devoid of p53 mutations Supplementary Information, Fig. S2^{24,26–28}, and that p53 ablation does not

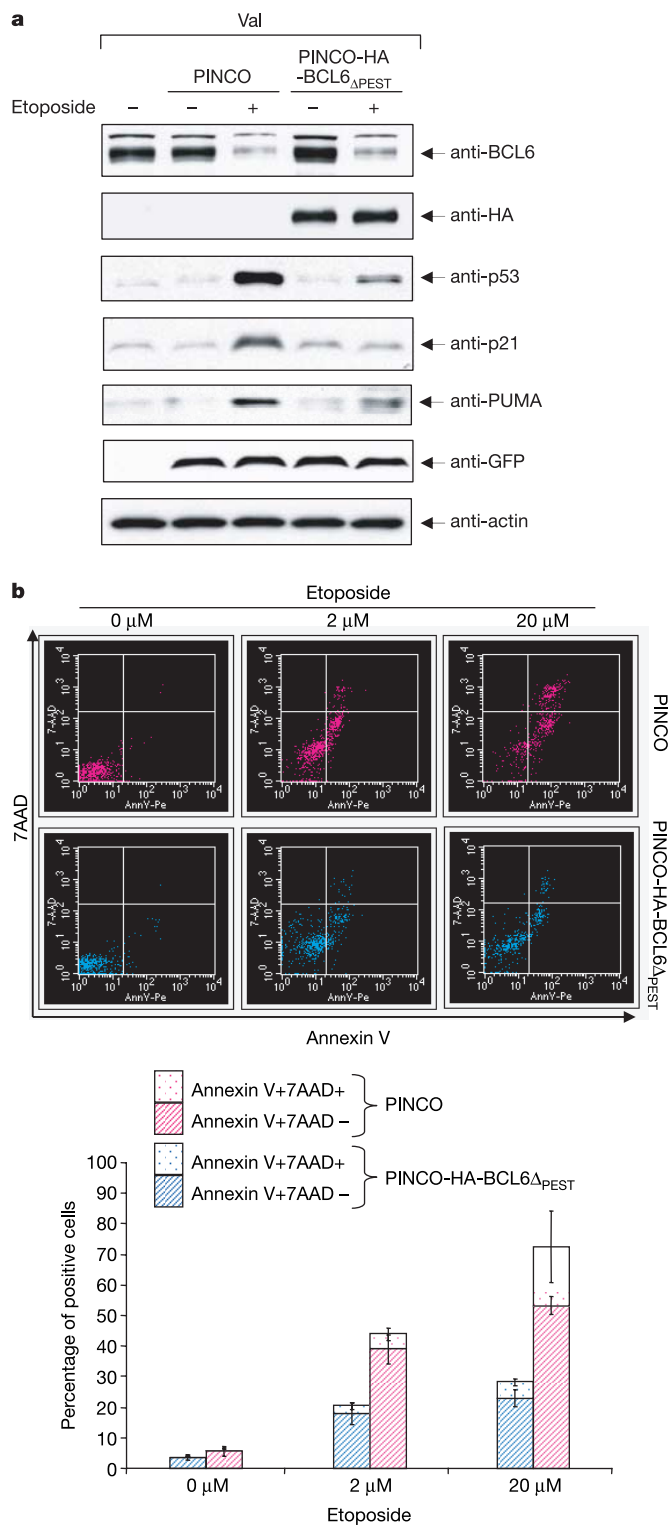


Figure 4 Constitutive expression of BCL6 protects from DNA damage-induced apoptosis in B cells. **a**, Expression of endogenous BCL6, exogenous BCL6 (HA), p53, p21 and PUMA in transduced Val cells carrying control PINCO or PINCO-HA-BCL6 Δ PEST vector upon treatment with or without etoposide at 20 μ M for 6 h. Expression of GFP and β -actin was analysed as controls. **b**, The experiments were performed as described in the Methods and the apoptotic effect was analysed by FACS using annexin V and 7AAD staining (upper panel). The lower panel shows the percentage of positive cells ($\pm$ s.d.) of three independent experiments.

affect lymphomagenesis in BCL6 transgenic mice (our unpublished results). Because the simultaneous inactivation of BCL6 and activation of p53 can be obtained pharmacologically both by acetylation²⁹ and by DNA damage (Fig. 3d, e), these results have implications for the treatment of DLBCL. □

Methods

Plasmids

pMT2T-HA-BCL6 and its derivatives have been previously described²⁹. The p53n-luc was constructed by inserting a PCR-amplified genomic region of human p53 promoter (−3,123 to +396) upstream of luciferase gene in pGL3 basis vector (Promega). The p53n-luc derivatives carrying a point mutation at BCL6 binding sites were generated using the QuickChange site-directed mutagenesis kit (Stratagene). PINCO and PINCO-HA_{ΔPEST} retroviral vectors have been previously described⁵. The pSuppressor viral vector was obtained from Imgenex.

Cell lines and cell culture

The amphotropic packaging cell line Phoenix, human embryonic kidney 293 cells and its derivative 293T cells were maintained in DMEM supplemented with 10% FBS and antibiotics. Ramos cells and DLBCL Val cells were maintained in IMDM supplemented with 10% FBS and antibiotics.

Chromatin immunoprecipitation assay

The ChIP assays were performed essentially as previously described^{5,6}. PCR amplification was performed in chromatin immunoprecipitated fragments using different oligonucleotide pairs: fragment A (5′-TCAAGTGTCTGCACACCTC-3′ and 5′-GAAGAGAGACTAAGTGGAG-3′), fragment B (5′-CTGAATCCTTGAGGGAAGTAG-3′ and 5′-GCACCAACCTGAGAACTTC-3′), fragment C (5′-TGATAAGGGTTGTGAAGGAG-3′ and 5′-ATATGAAGGGTGAAGGAAG-3′) and fragment D (5′-TGGCTCCATTCATTAAGTGGAG-3′ and 5′-TCCTGCCACTTCTGATGGAG-3′). Fragments containing the BCL6 binding site in the BCL6 first exon were detected by PCR amplification as described⁶.

Transient transfection and reporter assays

293T cells were transiently transfected using the calcium-phosphate precipitation method and the luciferase reporter assays were performed as previously described^{17,29}. The pRL-TK vector was also co-transfected to control for transfection efficiency. Each transfection was done in duplicate and luciferase activities were measured 48 h post-transfection using the dual-luciferase reporter assay kit (Promega) according to the manufacturer's protocol. Normalized values are reported as the mean ± s.d. from triplicate transfections.

Retroviral infections

Retroviral transduction into B cell lines was performed as previously described^{5,29}. Briefly, the amphotropic packaging cell line Phoenix was transiently transfected with PINCO, and PINCO-HA-BCL6_{ΔPEST} using the calcium-phosphate precipitation method, and viral supernatants were collected 48 h after transfection. For infection, Val cells (5 × 10⁴ ml^{−1}) were resuspended in the viral supernatant supplemented with 0.5 μg ml^{−1} polybrene and centrifuged for 1 h at 450g. Green fluorescent protein (GFP) expression in Val cells was analysed by flow cytometry using a FACSCalibur (Becton Dickinson) and the transduced GFP⁺ Val cells were selected by cell sorting using FACStar (Becton Dickinson). The retroviral transduction of siRNA vectors into Ramos cells was performed according to the manufacturer's protocol (Imgenex), following the selection with G418 for 10–13 days, and clones were isolated for analysis.

siRNA-mediated inhibition of BCL6 synthesis

To generate BCL6 siRNA viral vectors, double-stranded oligonucleotides coding for siRNA that specifically target BCL6 transcript (BCL6 siRNA-1: 5′-GTCGAGACATCTTGACTGA-3′ and BCL6 siRNA-2: 5′-GACACGGATCTGAGAATCTC-3′) were cloned into pSuppressor vector according to the manufacturer's protocol. Vectors containing the inserts were verified by DNA enzymatic digestion and finally confirmed by DNA sequencing. The packaging retroviral vector pCL-Ampho and pCL-MFG-LacZ vector for controlling infection efficiency were also provided by Imgenex.

Immunoblotting and antibodies

Total cell lysis immunoblotting was performed as previously described²⁹. Antibodies against BCL6 (N3, C19 and D8), p53 (DO-1), p21 (C19) and OPG were purchased from Santa Cruz Biotechnology. Antibodies against the following proteins were obtained: Puma (Oncogene Research Products), GFP (Pharmingen), β-Actin (Sigma) and haemagglutinin (Roche).

RNA analysis

Total RNA extraction and polymerase chain reaction with reverse transcription (RT–PCR) analysis were performed as described previously³. Quantitative real-time RT–PCR was performed with the LightCycler and SYBR Green system (Roche) and the LightCycler Software 3 (Roche) was used for data analysis. The following oligonucleotide primers were used: p53, 5′-GCCCAACAACACCACTCT-3′ and 5′-CCTGGGCATCCTTGAGTTCC-3′; GAPDH, 5′-GAGTCAACGGATTGGTCTG-3′ and 5′-GACAAGCTTCCCGTTCTCAG-3′.

Apoptosis assay

Val cells were infected with PINCO, and PINCO-HA-BCL6_{ΔPEST} and sorted GFP⁺ cells were treated with etoposide at the indicated doses for 6 h, then replaced with fresh new medium and the apoptotic effect was measured 24 h later using annexin-V and 7AAD staining. Samples were subjected to flow cytometric analysis with FACSCalibur (Becton Dickinson). The quantitative analysis of the percentage of positive cells was reported from three independent experiments.

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Regulation of cellular response to oncogenic and oxidative stress by *Seladin-1*

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Expression of multiple oncogenes and inactivation of tumour suppressors is required to transform primary mammalian cells into cancer cells^{1–3}. Activated Ha-RasV12 (Ras) is usually associated with cancer, but it also produces paradoxical premature senescence⁴ in primary cells by inducing reactive oxygen species⁵ followed by accumulation of tumour suppressors p53 and p16^{INK4a} (ref. 4). Here we identify, using a direct genetic screen, *Seladin-1* (also known as *Dhcr24*) as a key mediator of Ras-induced senescence. Following oncogenic and oxidative stress, *Seladin-1* binds p53 amino terminus and displaces E3 ubiquitin ligase Mdm2 from p53, thus resulting in p53 accumulation. Additionally, *Seladin-1* associates with Mdm2 independently of p53, potentially affecting other Mdm2 targets. Ablation of *Seladin-1* causes the bypass of Ras-induced senescence in rodent and human fibroblasts, and allows Ras to transform these cells. Wild-type *Seladin-1*, but not mutants that disrupt its association with either p53 or Mdm2, suppresses the transformed phenotype. The same mutants are also inactive in directing p53-dependent oxidative stress response. These results show an unanticipated role for *Seladin-1*, previously implicated in Alzheimer's disease⁶ and cholesterol metabolism⁷, in integrating cellular response to oncogenic and oxidative stress.

We decided to search for regulators of oncogene-induced senescence using a targeted genetic screen (Fig. 1a) with a genetic suppressor element (GSE)⁸ complementary DNA library prepared from Rat1 cells. We expected that inactivation of genes essential for Ras-induced senescence by GSE sequences would allow cells to escape senescence, continue cell division and eventually form transformed cell colonies in the presence of ectopic expression of an activated Ras allele (Ha-RasV12). By using this screen, we isolated multiple independent antisense GSE sequences corresponding to a broadly expressed oxidoreductase gene (Fig. 1b, Supplementary Fig. 1), initially identified in plants (*DIMINUTO/DWARF1*)⁹ with the human orthologue originally described in neuronal cells (*Seladin-1*)⁶, and later shown to encode 3 β -hydroxysterol- Δ^{24} -reductase activity (*DHCR24*)⁷, further referred to here as *Seladin-1*.

To confirm the role of *Seladin-1* in senescence-inducing Ras signalling, early passage rat embryo fibroblasts (REFs) were infected with retrovirus expressing *Seladin-1* GSE (pBABEpuroR1-4; Supplementary Fig. 1). This resulted in a significant reduction of *Seladin-1* messenger RNA (not shown) and protein levels (up to 90%; Fig. 1c, left panel), with no apparent effect on cell growth or morphology (Fig. 1c, right panel). Upon introduction of Ras through retroviral infection, REF cells co-infected with the control vector (pBABEpuro) were arrested in 6–10 days, as expected⁴. The 'flat' cellular morphology and positive staining of these cells in senescence-associated β -galactosidase (SA- β -gal) assay¹⁰ indicated that they were senescent (Fig. 1d, upper left). In contrast, upon Ras introduction, REF cells with *Seladin-1* knockdown continued to proliferate, exhibited a 'condensed' cell morphology, and were not stained in SA- β -gal assay (Fig. 1d, upper right panel). Furthermore,

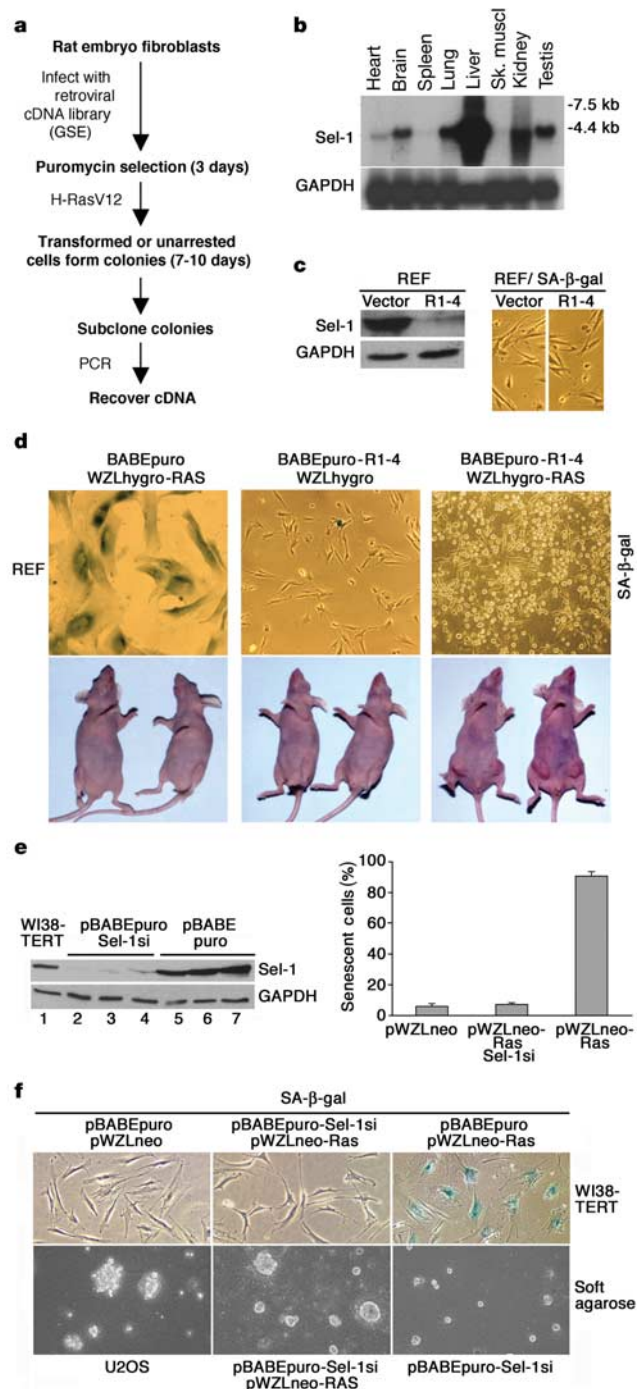


Figure 1 Involvement of *Seladin-1* in Ras-induced premature senescence. **a**, A genetic screen for regulators of Ras-induced premature senescence. GSE, genetic suppressor element. **b**, *Seladin-1* expression in adult mouse tissues (northern). GAPDH, loading control. kb, kilobases. sk., skeletal. **c**, Left: western blot of *Seladin-1* protein in rat embryo fibroblasts (REFs) and REFs expressing *Seladin-1* GSE (pBABEpuro-R1-4). Right: SA- β -gal staining of REFs infected with vector pBABEpuro or pBABEpuro-R1-4. **d**, SA- β -gal staining of REFs infected with pBABEpuro or pBABEpuro-R1-4 and transduced with pWZLhygro or pWZLhygro-Ras (upper row); tumorigenicity in nude mice (lower row). **e**, Left: western blot of *Seladin-1* in naïve WI38-TERT cells (lane 1), three clones transduced with pBABEpuro-*Seladin-1*si (lanes 2–4) or pBABEpuro (lanes 5–7). Right: penetrance of the senescent phenotype (SA- β -gal assay). Values are the means+s.d. ($n=3$). **f**, Upper row: SA- β -gal staining of WI38-TERT cells transduced with either empty pBABEpuro (left and right) or pBABEpuro-*Seladin-1*si (middle) following transduction with pWZLneo-RasV12 (middle and right) or with pWZLneo vector (left). Lower row: anchorage-independent growth in soft agar of U2OS cells (left), WI38-TERT cells transduced with pBABEpuro-*Seladin-1*si plus pWZLneo-HaRasV12 (middle) and pBABEpuro-*Seladin-1*si alone (right).

these cells formed tumours within two weeks when inoculated into nude mice (5/5 cases), indicating that they were oncogenically transformed (Fig. 1d, bottom right).

As rodent and human cells behave differently with regards to oncogenic transformation^{4,11}, we investigated whether *Seladin-1* would have a similar effect on Ras-induced senescence in human cells. We used stable RNAi (RNA interference)¹² to silence *Seladin-1* expression in human WI38 fibroblasts that were immortalized by the ectopic telomerase (TERT) expression. Western blot analysis indicated that Seladin-1 protein expression was abrogated in WI38-TERT cells by *Seladin-1* siRNA (small interfering RNA, pBABepuro-Seladin-1si; Fig. 1e, and Supplementary Methods).

When Seladin-1-ablated WI38-TERT cells were transduced with Ras, similar phenotype was observed as in *Seladin-1* knockdown REFs (Fig. 1e, f and Supplementary Results), indicating that *Seladin-1* is involved in regulating Ras-induced transformation and senescence both in human and rodent cells.

Ras-induced senescence is typically associated with activation of tumour suppressors p53, p16^{INK4a} and, in mouse cells, p19^{ARF} (ref. 4, 13). Intriguingly, while Ras-mediated p16^{INK4a} induction was unaffected in Seladin-1-ablated WI38-TERT cells, silencing of *Seladin-1* prevented Ras-induced p53 accumulation (Fig. 2a). p14^{ARF} shows only slight response to oncogenic Ras, which is in line with previous observations^{14,15}. This suggests that Seladin-1 is

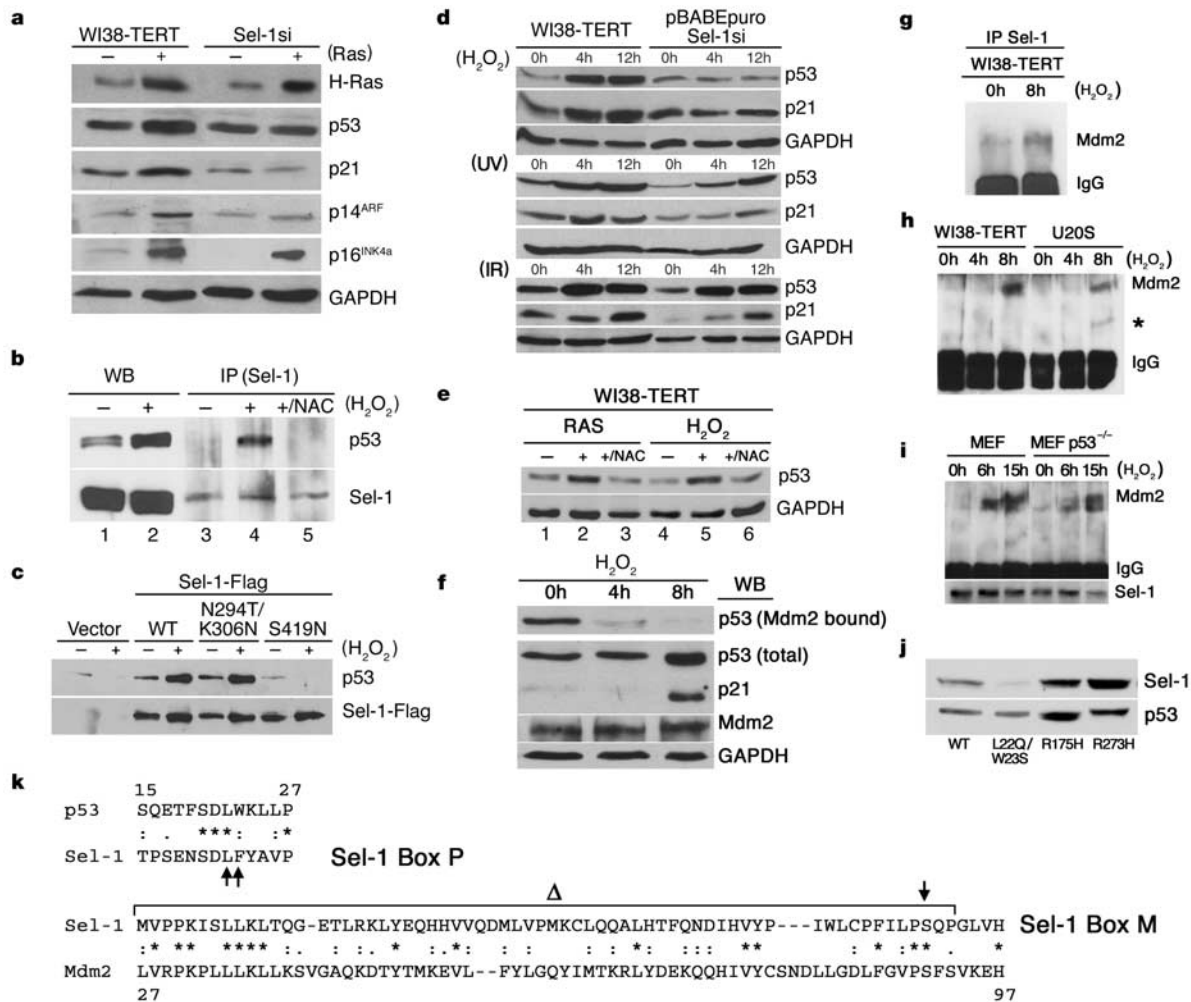


Figure 2 Effect of Seladin-1 on cellular response to Ras and oxidative stress.

a, b, Western blot (WB) analysis; **a**, WI38-TERT/pBABepuro and WI38-TERT/pBABepuro-Seladin-1si cells with (+) or without (–) ectopic Ha-RasV12 (Ras) transduction by WZLneoRasV12 retrovirus; **b**, Expression analysis of p53 and Seladin-1 in whole cell extracts (lanes 1 and 2) and Seladin-1 immunoprecipitates (IP; lanes 3–5) in the presence (+) (lanes 2, 4, 5) or absence (–) (lanes 1, 3) of H₂O₂ treatment. Lane 5, cells treated with N-acetylcysteine (NAC). **c**, Effect of Seladin-1 mutants on p53 binding in PA-1 cells. Immunoprecipitates from cells expressing empty pcDNA3 (Vector), wild-type Seladin-1 (WT), mutants N294T/K306N and S419N before (–) and after (+) treatment with H₂O₂, then blotted with p53 antibody. Anti-Flag shows the recombinant Seladin-1 variants expression. **d**, p53 and p21 in WI38-TERT and WI38-TERT expressing pBABepuro-Seladin-1si. H₂O₂ treatment (upper panel), UV irradiation (middle panel), or γ -irradiation (lower panel). **e**, Western blot analysis of p53 levels in naïve WI38-TERT cells or WI38-TERT ectopically expressing Ha-RasV12, in the presence/absence of H₂O₂ and NAC as indicated. **f**, Top panel; anti-p53 western blot after IP with anti-Mdm2 antibodies of

extracts from WI38-TERT cells treated with H₂O₂. Other panels; direct western blot of the same cell extracts with anti-p53, p21, Mdm2 and GAPDH antibodies. **g–i**, Seladin-1/Mdm2 IP-western blot of WI38-TERT (**g**), WI38-TERT and U2OS (**h**), and MEF and p53^{-/-} MEF cells (**i**) following H₂O₂ treatment as indicated. Asterisk in **h** indicates the 60-kDa Mdm2 fragment expressed in U2OS cells. Lower panel in **i** corresponds to same Seladin-1 IP re-blotted with anti-Seladin-1 antibodies. **j**, Immunoblot with anti-Seladin-1 antibodies of anti-Flag IPs (top panel) or direct western blot with anti-Flag antibodies of whole cell extracts obtained following PA-1 cell transfection with the appropriate Flag-tagged p53 mutants. **k**, Homology between the Mdm2 binding region of p53 and the P-box of Seladin-1, and the p53 binding region of Mdm2 and the M-box of Seladin-1. Alignment was obtained using ClustalW²⁷ (<http://www.ebi.ac.uk/clustalw/#>). Up arrowheads, p53 or Seladin-1 mutations inactivating both p53–Seladin-1 and p53–Mdm2 interaction (for p53 mutants) or Seladin-1–Mdm2 interaction (for Seladin-1 mutants). Δ , Deletion of the M-box from Seladin-1. Down arrowhead, S419N Seladin-1 mutant.

an essential mediator of Ras-induced p53 response. Premature senescence initiated by Ras depends on the increase of intracellular reactive oxygen species (ROS)⁵. Accordingly, scavengers of hydrogen peroxide (H₂O₂), a potent ROS producer, rescue Ras-induced senescence. Moreover, sublethal doses of H₂O₂ provoke a senescence-like phenotype in human fibroblasts¹⁶.

On these grounds, we sought to investigate whether Seladin-1 is integrated in Ras-initiated signalling downstream of ROS. Sublethal doses of H₂O₂ rapidly induced p53 and its downstream target, p21^{Cip1/Waf1}, in control cells (Fig. 2d, top panel). In contrast, H₂O₂ treatment did not cause a significant change in p53 and p21^{Cip1/Waf1} protein levels in *Seladin-1* knockdown cells, indicating that Seladin-1 is required for p53-mediated cellular response to ROS.

Moreover, we found that Seladin-1 is upregulated in response to both oxidative stress and Ras activation. Seladin-1 was induced in a time-dependent manner in WI38-TERT cells following H₂O₂ treatment, reaching the maximum level in 10–12 h (Fig. 3c). Similarly, Seladin-1 levels increased in response to Ras expression (Fig. 3d). Intriguingly, we found that Seladin-1 became associated with p53 in

response to both H₂O₂ treatment (Fig. 2b) and Ras activation (Supplementary Fig. 2). In this context, the hydrogen peroxide scavenger N-acetylcysteine (NAC) prevented accumulation of p53 (ref. 5 and Fig. 2e, top panel) and abrogated Seladin-1–p53 interaction (Fig. 2b). This suggests that increases in intracellular ROS trigger p53 and Seladin-1 interaction.

Immunolocalization studies indicated that Seladin-1, normally confined to the perinuclear cytoplasmic region (ref. 6 and Fig. 3a, left column), concentrated in the nuclei after oxidative challenge (Fig. 3a, middle column and Supplementary Fig. 3). Similar changes in Seladin-1 subcellular localization were also observed in WI38-TERT cells expressing a constitutively active Ras allele (Fig. 3a, right column and 3b). Treatment of these cells with NAC inhibited Seladin-1 induction (Fig. 3d) and its nuclear translocation (Fig. 3f, bottom panel), suggesting that Ras-promoted translocation or nuclear retention of Seladin-1 was mediated by ROS signalling.

Seladin-1 levels and localization were mostly unaffected by mild doses of UV or ionizing radiation (IR; here γ -irradiation), indicating that the cellular response to these stresses does not seem

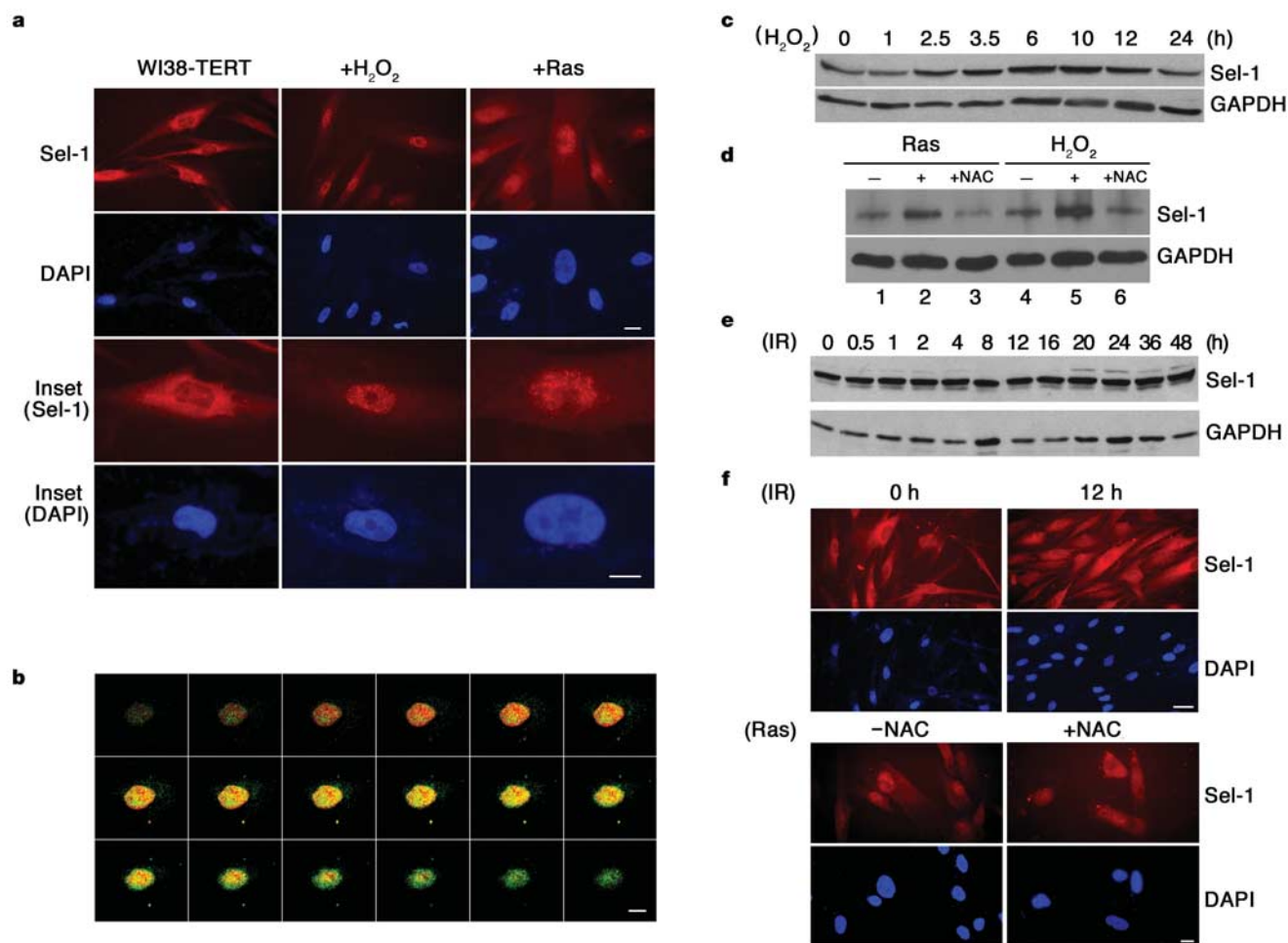


Figure 3 Regulation of Seladin-1 in response to different stimuli.

a, b. Immunofluorescence staining of: **a**, Seladin-1 in WI38-TERT cells (left column), H₂O₂-treated cells (10 h after treatment; middle column) and pWZLneo-Ras-infected cells (10 days after infection; right column). Seladin-1 was stained with rhodamine (red), and nuclei counterstained with DAPI (blue). **b**, Seladin-1 in WI38-TERT cells transduced with Ras. Confocal microscopy on progressive cell sections showing Seladin-1 (Alexa 488, green), DNA (propidium iodide, red) and co-localization, yellow. **c–e**, Western blot analysis of: **c**, Seladin-1 in WI38-TERT cells before and after H₂O₂ treatment. **d**, Seladin-1

in WI38-TERT cells transduced with pWZLneo (lane 1), pWZLneo-Ras alone (lane 2) or together with NAC treatment (lane 3), naïve WI38-TERT cells (lane 4), WI38-TERT cells treated with H₂O₂ (lane 5) and with H₂O₂ and NAC together (lane 6) as in Fig. 2c.

e, Seladin-1 in WI38-TERT cells before (0 h) and after γ -irradiation (0.5–48 h).

f, Immunofluorescence staining of Seladin-1 in WI38-TERT cells before and 12 h after γ -irradiation (upper), and in WI38-TERT/pWZLneo-Ras cells without and with NAC treatment (lower). Scale bars, 10 μ m. GAPDH, loading control.

to require Seladin-1 (Fig. 3e and f). Accordingly, Seladin-1 ablation failed to prevent UV- and IR-induced p53-dependent growth arrest (Figs 2d, 4e). Taken together, these results support a role of Seladin-1 specifically in the p53 response to oncogenic and oxidative stress which might be determined by the nature of damage caused by different stresses^{17,18}.

To gain further insight into the mechanism of the Seladin-1 effect on p53, we prepared p53 mutants and tested their interaction with Seladin-1 in co-immunoprecipitation (IP) assays. Interestingly, N-terminal p53 mutant(s), deficient in p53–Mdm2 interaction, also lost the ability to bind Seladin-1, suggesting that Seladin-1 associates with p53 at the Mdm2 binding site of the latter (Fig. 2j). In contrast, mutants of the p53 DNA binding domain retain interaction with Seladin-1 (Fig. 2j). Surprisingly, we also observed an association between Seladin-1 and Mdm2 (Fig. 2g–i) that was more pronounced in extracts from cells subjected to oxidative stress (Fig. 2g–i). Moreover, Seladin-1–Mdm2 interaction was independent of p53 and p14^{ARF}, since it was observed also in p53 null (MEF p53^{−/−}) and p14^{ARF} null cells (U2OS) under similar conditions (Fig. 2h, i). These results suggest that Seladin-1 might affect p53 by interfering with Mdm2-mediated p53 regulation.

To test this hypothesis, we prepared bacterially expressed

Flag-p53, Seladin-1 and Mdm2 (Supplementary Fig. 7) and found that, first, Seladin-1 binds p53 and displaces Mdm2 from p53 *in vitro* (Fig. 4c). Second, recombinant Seladin-1 inhibits *in vitro* ubiquitination of Flag-p53 by Mdm2 (Fig. 4d). Finally, levels of Mdm2-associated p53 decrease during oxidative stress *in vivo* (Fig. 2f, top panel) despite concomitant increase in overall p53 and stable Mdm2 levels in the same experiments (Fig. 2f).

Sequence analysis revealed that Seladin-1 contains two adjacent regions of homology with the Mdm2-binding site on p53 (Box P) and with the p53-binding site on Mdm2 (Box M; Fig. 2k and Supplementary Fig. 5). The significance of these sites for Seladin-1–p53 and Seladin-1–Mdm2 interactions is underlined by failure of the appropriate Seladin-1 mutants to interact with p53 (Figs 2c, 4b) and Mdm2 (Supplementary Fig. 6), respectively. Additionally, the Seladin-1 N294T/K306N double mutation, which inactivates its intrinsic DHCR24 activity⁷, has no effect on Seladin-1–p53 interaction (Fig. 2c). Our data support a model where Seladin-1 directly inhibits Mdm2–p53 interaction following oxidative stress, causing a decrease in Mdm2-dependent p53 ubiquitination and degradation, and resulting in accumulation of active p53 and subsequent initiation of the cell cycle arrest. Thus, Seladin-1 shows features of a potential tumour suppressor involved in cellular response to

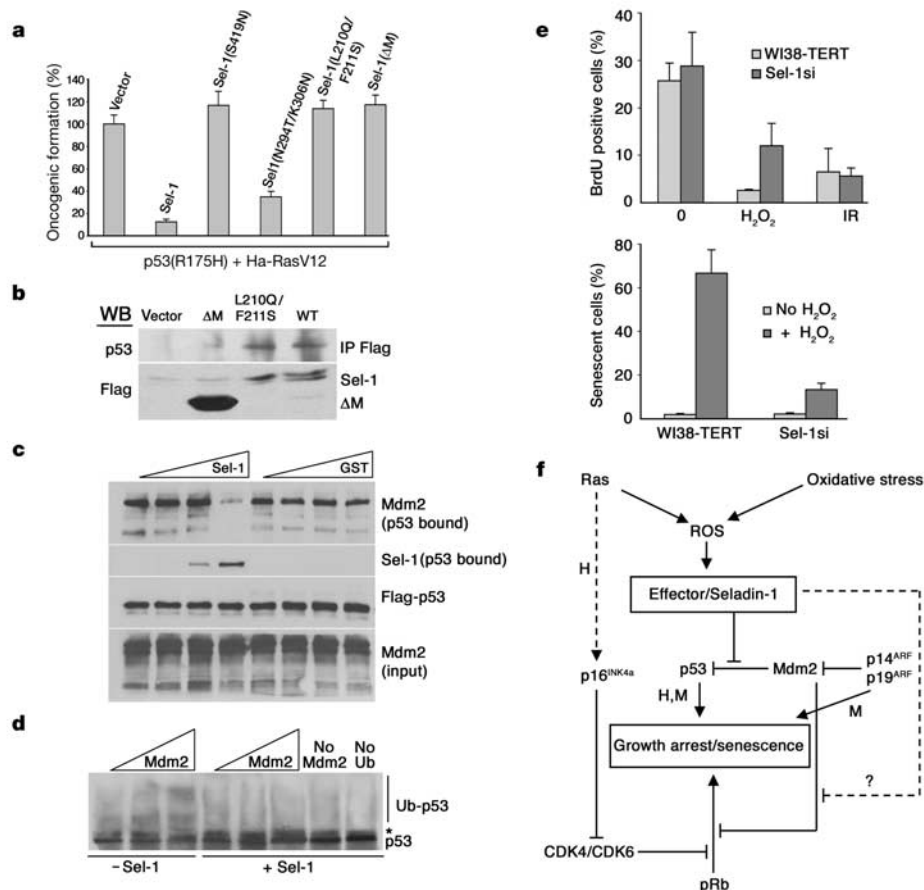


Figure 4 Seladin-1 as an inhibitor of p53–Mdm2 interaction, and as a potential tumour suppressor. **a**, Effect of Seladin-1 on REF transformation by p53(R175H) and Ha-RasV12. Normalized numbers of transformed foci for vector, wild-type Seladin-1 (Sel-1), and mutant Seladin-1 (Sel-1(S419N); Sel-1(N294T/K306N); Sel-1(L210Q/F211S) and Sel-1(ΔM) (as in Fig. 2k) are shown. The values were normalized to the number of foci observed on the control vector plates. The columns in the graph represent normalized average + s.d. ($n=3$). **b**, Effect of Seladin-1 mutations on its interaction with p53. Top panel, IP with anti-Flag, followed by immunoblot with anti-p53. Bottom panel, Flag immunoblot. P-box Seladin-1 mutant still binds p53. **c**, Inhibition of p53–Mdm2 interaction by the recombinant Seladin-1. Top row, immunoblot of Flag-p53-bound

Mdm2 following incubation with increasing amounts of GST-Seladin-1. Second row, p53-associated Seladin-1 from the same experiment detected with anti-Seladin-1 antibody. Bottom two rows, direct immunoblot of total Flag-p53 or Mdm2 at the conclusion of the experiment. **d**, Inhibition of Mdm2 ubiquitination of p53 *in vitro* by Seladin-1. Asterisk, unspecific band on top of p53. **e**, Effect of H₂O₂ treatment on DNA replication (BrdU incorporation, upper graph) and on senescence induction (SA-β-gal staining, lower graph) in WI38-TERT cells and cells expressing Seladin-1 siRNA (Seladin-1si). Results are mean percentages + s.d. ($n=3$). **f**, Model of Ras- and oxidative stress-initiated signalling pathway. H, human; M, mouse.

Ras/p53-mediated oncogenic signalling and oxidative stress.

Hence, Seladin-1 suppresses oncogenic foci formation promoted by co-expression of dominant negative p53 and oncogenic Ras in REFs (Fig. 4a). This activity depends on p53 and Mdm2 binding, since both M-box and P-box Seladin-1 mutants were ineffective (Fig. 4a, b; Fig. 2c and Supplementary Fig. 6). In contrast, abrogation of DHCR24 activity (N294T/K306N double mutant) had little effect on growth suppression (Fig. 4a) and p53 accumulation following oxidative challenge (Supplementary Fig. 4).

Inactivation of either p53 or p19^{ARF} in combination with an activated Ras allele suffices to transform rodent primary cells. In contrast, p53 mutation fails to overcome Ras-induced senescence in human primary cells^{4,19}. We observed that Seladin-1 ablation uniquely prevents human fibroblasts from arresting in response to Ras, while maintaining Ras growth stimulatory potential. This suggests that in addition to p53, Seladin-1 might affect other pathways relevant to Ras-mediated premature senescence in human cells. Accordingly, since Seladin-1 also interacts with Mdm2 independently of p53, this suggests a possible effect on other Mdm2 targets, such as pRB^{20–24} (Fig. 4f). Additionally, since Seladin-1 displaces Mdm2 from p53, other p53 modifications catalysed by Mdm2²⁵ might also be affected by Seladin-1.

The similarity of the Seladin-1 response to Ras and oxidative stress suggests that Seladin-1 may unexpectedly serve as an important ROS effector in mammalian cells. Concordantly, we observed that cells with Seladin-1 knockdown continued to show BrdU incorporation (Fig. 4e, top panel), cell proliferation (Supplementary Fig. 8) and approximately fourfold less SA- β -gal staining (Fig. 4e, lower panel) after H₂O₂ treatment, suggesting an essential role for Seladin-1 in the oxidative stress response senescence program¹⁶. Interestingly, Ras activation and ROS generation were not affected by Seladin-1 (Supplementary Fig. 9), placing Seladin-1 downstream of Ras and ROS in premature senescence and oxidative stress response pathways (Fig. 4f).

Seladin-1 downregulation coincides with Alzheimer's disease⁶, but the molecular basis of this correlation remains unclear. The only biological activity of Seladin-1 described previously was 3 β -hydroxysterol- Δ ²⁴-reductase (DHCR24) that catalyses the reduction of desmosterol, the last step in cholesterol biosynthesis⁷. We show here that the DHCR24 activity of Seladin-1 is apparently not required for p53 binding and p53-dependent oxidative stress response (Fig. 2c and Supplementary Fig. 4). Nevertheless, two seemingly independent Seladin-1 functions affecting cholesterol and p53 might share some regulatory mechanisms. In fact, oxidation of cholesterol produces ROS²⁶ and this gives rise to a potential crosstalk between these two pathways, with Seladin-1 acting as a potential sensor and/or effector of oxidative stress.

Ras and tumour suppressors p53 and p14^{ARF}/p19^{ARF} are involved in oncogenesis, cellular senescence and ageing. Additionally, both oxidative stress and cholesterol metabolism have been implicated in ageing and degenerative disorders at the organismal level. Our data support the notion that Seladin-1 stands at the intersection of all these processes. □

Methods

Vectors and cell culture

Retroviral vectors pBABEpuro, pWZLhygro and pWZLneo were used to construct pBABEpuro-Seladin-1, pBABEpuro-R1-4 and pBABEpuro-Seladin-1si, pWZLhygro-H-rasV12 and pWZLneo-H-rasV12. pWZLhygro-hTERT was a gift from R. Weinberg. Retrovirus preparation, infection and selection were done as described⁴. Cell lines and growth conditions are described in Supplementary Methods.

GSE cDNA library screen

The retroviral GSE screen was performed as previously described⁸; details are given in Supplementary Methods. Antisense GSE sequences recovered from the screen were used to search against available NCBI databases to find the corresponding genes and their homologues in different species. Accession numbers in GenBank for the corresponding genes are: human *Seladin-1/DHCR24* cDNA D13463 (partial), AF261758 (full); genomic

clone AC009946; mouse cDNA AY039762; *Arabidopsis thaliana* DWF1 U12400; *Caenorhabditis elegans* homologue AF026214.

Senescence-associated β -galactosidase staining

The staining was performed essentially as described⁴. Cells were washed briefly with distilled water after staining and then examined under an inverted Olympus IX70 microscope.

Immunoprecipitation, western blot, cell growth and tumorigenesis

Cells were treated as described in Supplementary Methods.

siRNA inhibition of Seladin-1 expression

Two oligonucleotides were synthesized and annealed: 5'-GATCCCTCGGAAGGAGCA GGGTAGCATTCAGAGATGCTACCTGCTCCTTCCA TTTTGGAAA-3' and 5'-AGCTTTTCCAAAAATGGAAGGAGC AGGGTAGCATCTCTTGAATGCTACCTGCTCCTTCCAGGG-3' (siRNA target recognition sequence is underlined). The annealed double-strand oligonucleotide was then inserted into the *Bgl*II-*Hind*III sites of pSUPER¹². To improve the RNAi efficiency, the siRNA expression cassette (*Bam*HI-*Sal*I fragment) was removed from pSUPER-Seladin-1si and cloned into *Bam*HI-*Sal*I digested pBABEpuro. The resultant pBABEpuro-Seladin-1si was used to generate retrovirus and infect WI38-TERT cells. Puromycin selection was applied for three days to generate cells with a stable inhibition of Seladin-1.

Seladin-1, mutants, expression, purification, ubiquitination assays

Detailed procedures are described in Supplementary Methods.

BrdU incorporation assay

The assay was done using the *in situ* Cell Proliferation Kit, FLUOS, following the instructions (Roche). Cells were cultured on coverslips, and BrdU labelling was for 4 h. The BrdU signal was examined under an inverted Olympus IX70 microscope equipped with proper fluorescence filters.

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Poly(ADP-ribose) is required for spindle assembly and structure

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The mitotic spindle is typically thought of as an array of microtubules, microtubule-associated proteins and motors that self-organizes to align and segregate chromosomes¹. The major spindle components consist of proteins and DNA, the primary structural elements of the spindle¹. Other macromolecules including RNA and lipids also associate with spindles, but their spindle function, if any, is unknown. Poly(ADP-ribose) (PAR) is a large, branched, negatively charged polymeric macromolecule whose polymerization onto acceptor proteins is catalysed by a family of poly(ADP-ribose) polymerases (PARPs)². Several PARPs localize to the spindle in vertebrate cells, suggesting that PARPs and/or PAR have a role in spindle function². Here we show that PAR is enriched in the spindle and is required for spindle function—PAR hydrolysis or perturbation leads to rapid disruption of spindle structure, and hydrolysis during spindle assembly blocks the formation of bipolar spindles. PAR exhibits localization dynamics that differ from known spindle proteins and are consistent with a low rate of turnover in the spindle. Thus, PAR is a non-proteinaceous, non-chromosomal component of the spindle required for bipolar spindle assembly and function.

PAR polymers are the product of post-translational modifications and are required for normal cell division; in *Drosophila*, PARP knockouts are embryonic lethal³. PAR is polymerized by PARPs onto acceptor proteins using NAD⁺ as substrate², and the concentration, length and extent of PAR branching are regulated by a balance of activities of the PARPs and poly(ADP-ribose) glycohydrolase (PARG), a highly specific, processive endo- and exoglycosidase⁴. Most PARPs localize to the spindle: PARP1 and PARP2 localize to centromeres and interact with CENPA, CENPB and Bub3 (ref. 5); VPARP localizes throughout the spindle⁶; PARP1 and PARP3 associate with centrosomes²; and tankyrase1 and 2 are found at chromosomes and spindle poles⁷, where they interact with the spindle pole protein NuMA⁸. Cellular PAR concentrations

markedly increase during metaphase and anaphase–telophase owing to PARP activity⁹. Together, these observations suggest that PARPs, or PAR itself, might be important in spindle function.

We examined PAR localization in metaphase spindles by staining isolated spindles—assembled in cycled *Xenopus* egg extract and in somatic cells—using three different antibodies against PAR, and varied fixation conditions (Fig. 1a, b). In all cases, PAR was filamentous and punctate in appearance, co-localized with spindle microtubules, and was enriched at spindle poles and kinetochores (Fig. 1a, b; see also Supplementary Fig. S1). PAR filaments were most evident without fixation and became more punctate after fixation, suggesting that traditional fixation methods developed for proteins perturb PAR localization. PAR localization to the kinetochore is particularly interesting in light of data demonstrating PARP1 recruitment to metaphase centromeres¹⁰, suggesting a role for PARP1 or PAR in centromere/kinetochore function.

The staining pattern in metaphase cells was similar to isolated *Xenopus* spindles except for a more pronounced enrichment at the poles (Fig. 1b). At the poles, PAR was primarily associated with spindle pole material rather than centrosomes, and spindle pole staining was similar to the localization pattern seen for tankyrase1 (ref. 11). Interestingly, PARG also co-localized with spindle microtubules in BSC1 and HeLa cells (Fig. 1c). This co-localization of PARPs, PAR and PARG with spindle microtubules suggests that one or more PARP(s) and PARG may actively regulate PAR levels in the spindle.

To determine whether PAR is enriched in the metaphase spindle, we compared the amount of spindle-associated PAR to total mitotic frog egg extract (CSF) by isolating spindles from *Xenopus* extract, and resolving equivalent concentrations of purified spindles and CSF on SDS polyacrylamide gels. Immunoblotting with anti-PAR antibodies showed a ~10-fold enrichment of PAR in the spindle relative to surrounding cytoplasm (Figs 1d and 2c). Most of the poly(ADP-ribosyl)ated (PARsylated) proteins were between ~97 and ~200 kDa. These may represent a single, highly modified protein, or a population of PARsylated proteins of varying sizes (Figs 1d and 2c). PAR and tubulin concentrations were unaffected by detergent treatment before spindle isolation, showing that PAR directly associates with the spindle rather than the membranes that co-purify with isolated spindles.

To ask whether PAR has an important function in the spindle, we assembled spindles in the presence of X-rhodamine-labelled tubulin in extracts containing replicated chromosomes¹² (cycled spindles), and perturbed PAR polymer by adding exogenous PARG or high concentrations of purified anti-PAR antibodies. We observed spindle structure using real-time, spinning-disk confocal microscopy (Fig. 2a) or examined fixed samples at defined time points (Fig. 2b, e). Notably, addition of 100 µg ml⁻¹ PARG resulted in the rapid breakdown of spindle structure (Fig. 2a; see also Supplementary Movie M1). Microtubules rapidly splayed outward from the spindle centre, after which the two half spindles appeared to become disconnected and the degree of anti-parallel microtubule overlap decreased markedly. This effect was specific to the hydrolytic activity of PARG, as simultaneous addition of 100 µM of the PARG inhibitor ADP-HPD¹³ blocked the phenotypic effect of PARG (Fig. 2b). We quantified the effect of PARG addition on both PAR and tubulin concentrations in the spindle by isolating treated and untreated spindles and blotting for PAR or tubulin (Fig. 2c). Addition of 100 µg ml⁻¹ PARG to assembled spindle reactions resulted in the complete hydrolysis of PAR polymer up to the detection limit of our testing, whereas simultaneous addition of 100 µM ADP-HPD partially protected PAR, especially at high molecular weight. Addition of 500 µg ml⁻¹ of affinity-purified anti-PAR antibody to cycled spindles phenocopied the effects of PARG addition (Fig. 2a; see also Supplementary Movie M2). In all cases extracts remained in mitosis, as demonstrated by the presence of condensed chromosomes. The phenotype of PAR hydrolysis was not

due to increased concentrations of soluble PAR, as addition of $500 \mu\text{g ml}^{-1}$ purified exogenous PAR had no effect on spindle structure (not shown). Taken together, these results suggest that PAR within the spindle is required for maintenance of bipolar spindle structure.

To determine whether PAR is required for the assembly of spindles, we added PARG before spindle assembly and analysed fixed samples 30 min after bipolar spindles had formed in untreated control reactions. Addition of $100 \mu\text{g ml}^{-1}$ PARG led to a complete

abrogation of spindle assembly—no bipolar spindles were identified in ten fields analysed at $40\times$ magnification. However, monopolar microtubule asters were present in association with normal mitotic chromosomes (Fig. 2b). Co-incubation of PARG and $100 \mu\text{M}$ ADP-HPD partially restored spindle assembly (Fig. 2b)—60 bipolar spindles were identified in ten random fields in two independent experiments. The presence of microtubule asters suggests that PAR is specifically required for bipolar spindle assembly. Furthermore, when we treated non-chromosomal, acen-

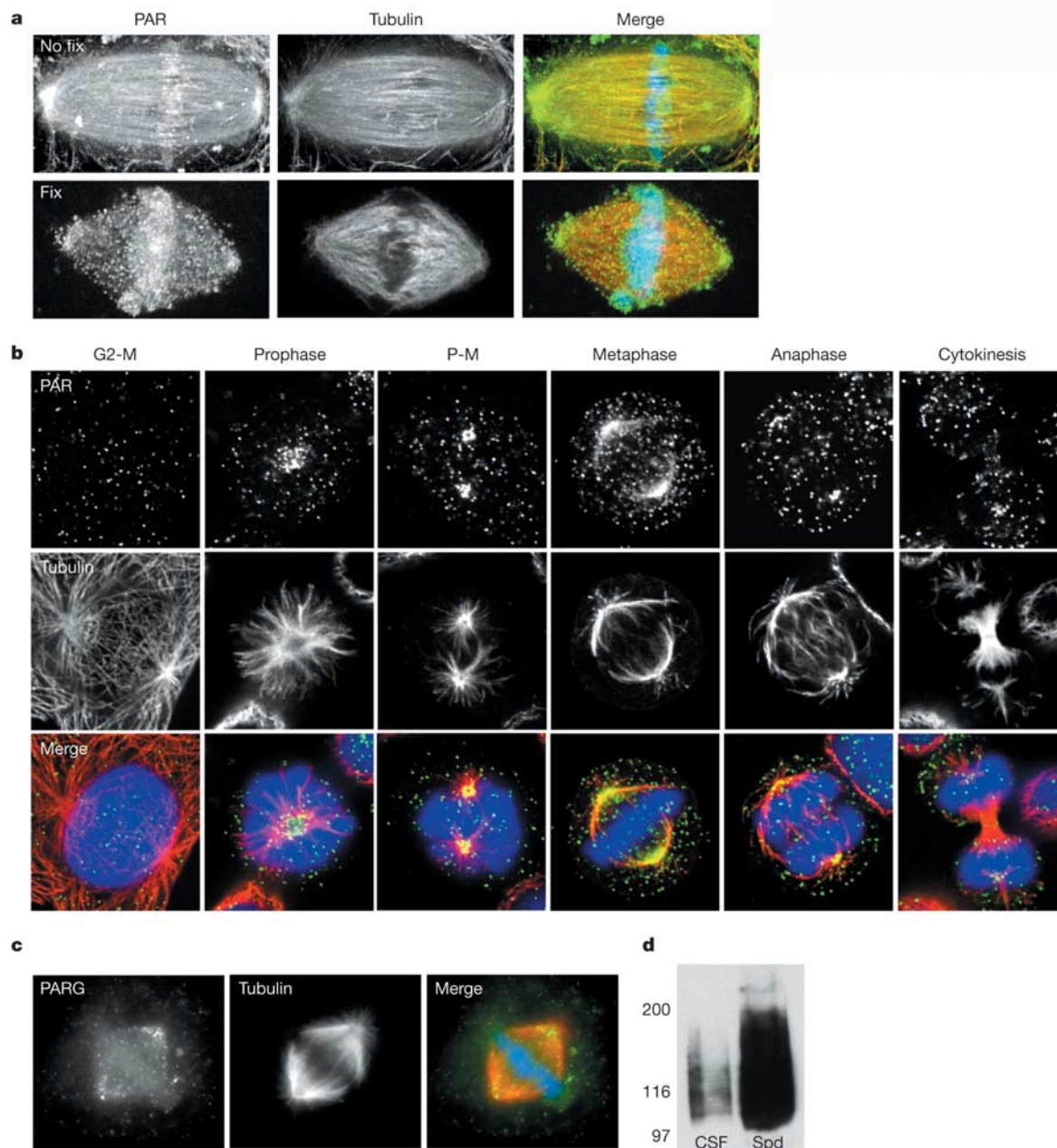


Figure 1 PAR is enriched in the spindle. **a**, PAR localization in purified *Xenopus* spindles. *Xenopus* egg extract spindles were isolated and stained for PAR, tubulin and DNA, without fixation (no fix), or after fixing with 4% paraformaldehyde (fix). PAR localizes throughout the spindle and is enriched at kinetochores and spindle poles (Supplementary Fig. S1). PAR appears filamentous when not fixed, and appears more punctate after fixation. In the merged panel, PAR is green, tubulin red and DNA is blue. **b**, Mitotic localization of PAR in somatic cells. HeLa, U2OS, LLPCK, XLK and BSC1 cells were fixed and stained for PAR, tubulin and DNA. All cell types exhibited similar staining patterns. HeLa cells during G2-M, prophase, prometaphase (P-M), metaphase, anaphase and cytokinesis stages of the cell

cycle are shown. PAR localizes to the mitotic spindle throughout mitosis and appears filamentous during metaphase, similar to the staining pattern seen in isolated *Xenopus* egg extract spindles, which are arrested in metaphase. In the merged panel, PAR is green, tubulin red and DNA is blue. **c**, PARG localizes to the mitotic spindle. HeLa cells were stained with antibodies against PARG and tubulin. PARG co-localizes with tubulin, as shown in the merged panel (PARG is green, tubulin red and DNA is blue). **d**, PAR is enriched in the spindle. Immunoblot of equivalent amounts of CSF extract or isolated spindles (Spd) probed with anti-PAR antibody. Molecular mass is marked along the left (kDa).

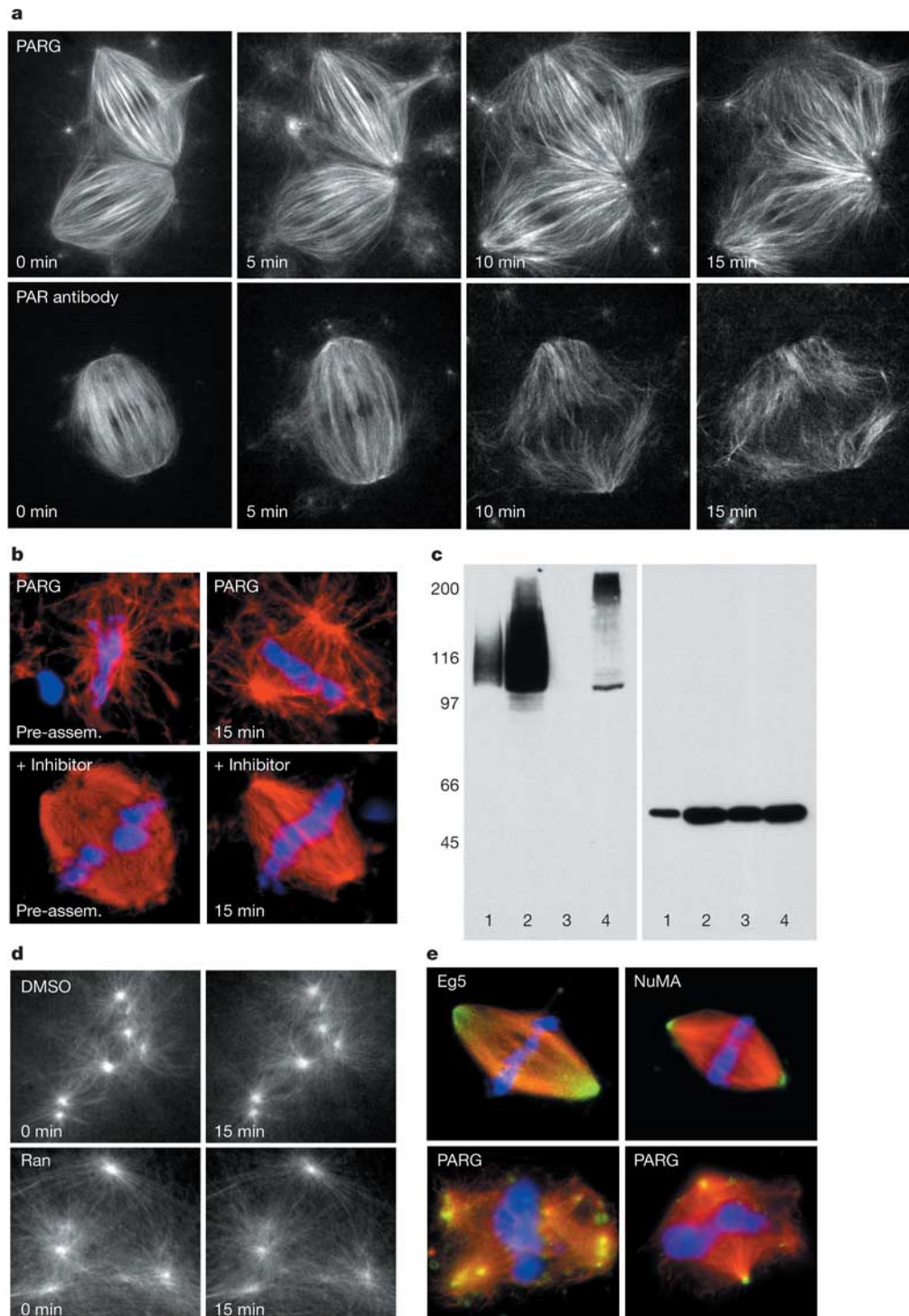


Figure 2 PAR is required for bipolar spindle assembly and function. **a**, PAR perturbation results in rapid loss of spindle structure. Cycled *Xenopus* egg extract spindles assembled in the presence of X-rhodamine-labelled tubulin were treated with $100 \mu\text{g ml}^{-1}$ PARG or $500 \mu\text{g ml}^{-1}$ purified anti-PAR antibody, and real-time images obtained every 30 s for 15 min on a spinning disk microscope. (See Supplementary Movie M1 (PARG addition) and Supplementary Movie M2 (PAR antibody addition) for full sequences.) **b**, PAR is required for spindle assembly and function. Addition of $100 \mu\text{g ml}^{-1}$ PARG for 2 h during (pre-assem.) and for 15 min after (15 min) spindle assembly both resulted in spindle monopoles with associated condensed chromosomes. These phenotypes are specific for PAR hydrolysis, as addition of $100 \mu\text{M}$ ADP-HPD (+inhibitor), a PARG inhibitor, rescues spindle bipolarity. Tubulin is red and DNA is blue. **c**, Quantification of PAR hydrolysis. Equal amounts of CSF extract (lane 1) or cycled spindles reactions were untreated (lane 2),

treated with $100 \mu\text{g ml}^{-1}$ PARG (lane 3), or $100 \mu\text{g ml}^{-1}$ PARG plus $100 \mu\text{M}$ ADP-HPD (lane 4) and probed for antibodies against PAR (left blot) or tubulin (right blot). PARG treatment (lane 3) results in complete hydrolysis of PAR, whereas simultaneous addition of ADP-HPD partially protected PAR (lane 4). Molecular mass is indicated along the left (kDa). **d**, PAR is not required for microtubule aster structure or assembly. A total of $100 \mu\text{g ml}^{-1}$ PARG was added 25 min before DMSO- or RanGTP-induced (Ran) aster assembly in CSF extract, and real-time images obtained every 30 s for 15 min. PARG addition had no effect on aster assembly or structure. **e**, Eg5 and NuMA localization after PAR disruption. Cycled *Xenopus* extract spindles were not treated (top) or were treated with $100 \mu\text{g ml}^{-1}$ PARG (bottom), isolated and stained for tubulin (red) and Eg5 or NuMA (green), two proteins required for spindle assembly. Eg5 and NuMA remained associated with the resulting structures (PARG), suggesting that PAR may not be required for their targeting to spindles.

trosomal asters—formed by addition of Ran-GTP¹⁴ or DMSO—with 100 $\mu\text{g ml}^{-1}$ PARG (Fig. 2d), neither the size nor the density of asters was affected. Thus, PAR has a specific role in organizing bipolar spindles, and is not required for microtubule nucleation or dynamics.

We next determined the effect of PAR disruption on the localization of proteins previously implicated in spindle assembly: that is, Eg5, a tetrameric kinesin motor protein required for spindle bipolarity¹⁵, and NuMA, a non-centrosomal component of the spindle poles required for pole focusing¹⁶. Importantly, Eg5 (ref. 15) and NuMA¹⁷ localize to both bipolar spindles and microtubule asters. We added 100 $\mu\text{g ml}^{-1}$ PARG to assembled cycled spindles containing X-rhodamine-labelled tubulin, purified the resulting structures through glycerol cushions, and stained for Eg5 or NuMA (Fig. 2e). Both proteins associated with the microtubule structures, suggesting that PAR is not required for their

targeting to spindles, although we cannot rule out more subtle interactions of these proteins with PAR.

Microtubule-associated spindle components including tubulin, microtubule-associated proteins and Eg5 have been shown to exchange rapidly between spindle-associated and cytoplasmic pools^{18–20}. PAR differs biochemically and biophysically from known spindle molecules and might exhibit unique localization dynamics. As a first approach to analysing dynamics, we measured the half-life of anti-PAR Fab fragments bound to spindles. Fab fragments generated from purified anti-PAR LP96-10 antibody were directly labelled with X-rhodamine. In addition, we directly labelled LP96-10 IgG and anti-PAR IgY with X-rhodamine and Alexa 488. All yielded similar localization patterns. Directly labelled anti-PAR Fab was added to cycled spindles, and its localization examined using real-time wide-field microscopy (Fig. 3a). These low concentrations of antibody, unlike the 50 $\times$ higher concentrations used earlier to disrupt spindle function, had no obvious effect on spindle morphology. To measure exchange between spindle-associated and cytoplasmic PAR, we concentrated spindles labelled with 10 $\mu\text{g ml}^{-1}$ X-rhodamine-labelled anti-PAR Fab by sedimentation, diluted them 1:20 in unlabelled extract, and measured the loss of fluorescence intensity relative to the initial time point. For comparison, we repeated the experiment using 10 $\mu\text{g ml}^{-1}$ X-rhodamine-labelled antibody against the microtubule-associated protein TPX2 (ref. 21). Labelled tubulin exchanged out of the spindle rapidly, as expected (not shown). TPX2 antibody exchanged out with a half-life of ~ 5 min, confirming that TPX2 is a dynamic spindle component. In contrast, PAR fluorescence did not decrease appreciably during the course of the dilution experiment (Fig. 3b), indicating a half-life longer than that of known spindle components.

Our data demonstrate a requirement for PAR in the assembly and structure of bipolar spindles, and help to explain PARP localization to the spindle. How might PAR function in the spindle? The best understood mechanism of PAR activity is regulation of polynucleosome activity during DNA damage: PARsylation of histones results in a conformational change in the polynucleosome leading to altered nucleosome–DNA interactions and changes in chromatin condensation²². Similarly, PARsylation of spindle proteins including microtubule-associated proteins and molecular motors may regulate their activity, and as a result, spindle assembly and function. Thus PAR may act as a molecular on/off ‘switch’ for spindle assembly. Phosphorylation, a hallmark of mitosis, increases the activity of PARP1 and tankyrase1 (refs 23, 24) and may regulate the activity of all PARPs. For example, PARP1 is inactive in oocytes, and PAR is not detectable; however, PARP activity is increased after phosphorylation of PARP1 by mitotic kinases, without any change in PARP protein concentrations²³. This suggests that phosphorylation may also regulate PARP activity during mitosis, explaining the increase in PAR concentration during mitosis⁹. We do not know the identity of PAR acceptors in the spindle aside from the PARPs themselves, as all known PARPs undergo auto(ADP-ribosyl)ation. One interesting candidate is NuMA, a spindle protein required for spindle pole assembly and recently shown to interact directly with tankyrase1 (ref. 8). PARsylation of NuMA may be required for spindle function.

Another possibility is that PAR has a structural role in the spindle. The size, branching, high negative charge and aromatic heterocycle functionality of PAR suggest that it could mediate charge–charge interactions with proteins in addition to covalent modification of acceptors. This would explain the disruption of spindle structure observed when large amounts of anti-PAR antibody are added. PARsylated proteins in the nucleus have been shown to interact with non-modified proteins through charge–charge interactions with PAR, interactions thought to mediate assembly of a nuclear matrix²⁵. A similar matrix may exist in the spindle, as proposed by the spindle matrix hypothesis²⁶. Finally, we note that PARP1 is a

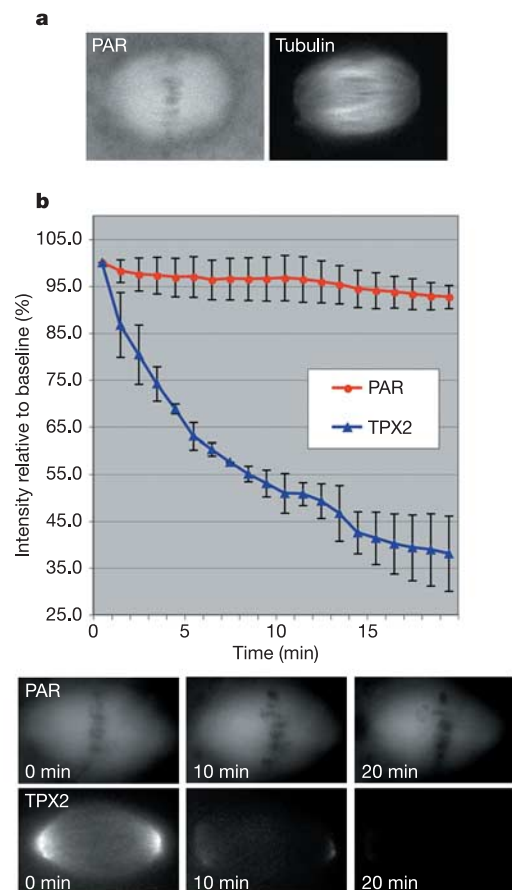


Figure 3 PAR exchange between the spindle and cytoplasm is slow. **a**, Real-time localization of PAR in the spindle. X-rhodamine-labelled anti-PAR Fab fragments were added to cycled spindles containing Alexa-488-labelled tubulin and visualized. PAR localization to the spindle largely overlaps with tubulin. **b**, Rate of PAR exchange between the spindle and cytoplasm. *Xenopus* egg extract spindles incubated with X-rhodamine-labelled anti-PAR Fab or anti-TPX2 antibodies were partially purified by sedimentation, diluted 1:20 into unlabelled mitotic egg extract and images taken every 30 s for 20 min using wide-field microscopy. The graph depicts the percentage relative fluorescence intensity with respect to time 0 at every minute for three separate experiments. PAR (red line) exhibits a slow rate of cytoplasmic exchange relative to TPX2 (blue line), longer than known spindle components. Error bars represent standard deviation of three independent experiments. Below are images of anti-PAR and anti-TPX2 antibodies at 0, 10 and 20 min.

common drug target²⁷, suggesting that the spindle-associated PARPs are potential cancer drug targets. □

Methods

Imaging and antibodies

Cycled *Xenopus* egg extract spindles were assembled as described¹² with or without X-rhodamine-labelled or Alexa-488-labelled tubulin, and isolated through 40% glycerol BRB80. For staining of non-fixed spindles, isolated spindles were processed for immunofluorescence in solutions containing 5% glycerol BRB80. For immunofluorescence and immunoblotting, polyclonal rabbit LP96-10 IgG was purchased from BD Biosciences, polyclonal chicken IgY anti-PAR antibodies from Tulip Biolabs, and monoclonal 10H from Trevigen. All anti-PAR antibodies were pre-adsorbed against BSA transferred to nitrocellulose. LP96-10 Fab fragments were generated using an Immunopure Fab preparation kit (Pierce Biotech). Anti-NuMA, TPX2 and Eg5 polyclonal rabbit antibodies were gifts from A. Groen and D. Miyamoto. Rabbit anti-PARG antibody was obtained from Oncogene Inc. Primary antibodies were directly labelled using Alexa 488 and X-rhodamine NHS esters (Molecular Probes) as per the manufacturer's instructions. Measurements of fluorescence intensity were calculated by background subtraction, then integration of fluorescence. Images were obtained using a Princeton Instruments CCD camera, controlled by Metamorph software (Universal Imaging Corp.). Spinning disk confocal microscopy movies were obtained using a Nikon TE2000U inverted microscope, a Perkin Elmer Ultraview spinning disk confocal head, and a Hamamatsu Orca ER cooled-CCD camera controlled using Metamorph software. Spindle reactions were imaged in open chambers²⁸, and images obtained every 30 s for 15 min.

Protein purification and biochemistry

Recombinant bovine PARG was expressed and purified as described²⁹. For quantification of PAR after PARG treatment, spindles treated with 100 µg ml⁻¹ PARG, 100 µg ml⁻¹ PARG plus 100 µM ADP-HPD, or untreated spindles were isolated as above and resolved on 8% SDS-PAGE gels, transferred to nitrocellulose, and probed with LP96-10 antibody. CSF was added for comparison.

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Structure of an auxilin-bound clathrin coat and its implications for the mechanism of uncoating

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Clathrin-coated pits invaginate from specific membrane compartments and pinch off as coated vesicles. These vesicles then uncoat rapidly once released. The Hsc70 molecular chaperone effects the uncoating reaction, and is guided to appropriate locations on clathrin lattices by the J-domain-containing co-chaperone molecule auxilin^{1–4}. This raises the question of how a local event such as ATP hydrolysis by Hsc70 can catalyse a global disassembly. Here, we have used electron cryomicroscopy to determine 12-Å-resolution structures of *in-vitro*-assembled clathrin coats in association with a carboxy-terminal fragment of auxilin that contains both the clathrin-binding region and the J domain. We have located the auxilin fragment by computing differences between these structures and those lacking auxilin (described in an accompanying paper⁵). Auxilin binds within the clathrin lattice near contacts between an inward-projecting C-terminal helical tripod and the crossing of two ‘ankle’ segments; it also contacts the terminal domain of yet another clathrin ‘leg’. It therefore recruits Hsc70 to the neighbourhood of a set of critical interactions. Auxilin binding produces a local

change in heavy-chain contacts, creating a detectable global distortion of the clathrin coat. We propose a mechanism by which local destabilization of the lattice promotes general uncoating.

The clathrin-uncoating activity of Hsc70 was originally identified by biochemical assays *in vitro*^{1–3}, but convincing demonstration of its *in vivo* function required the characterization of auxilin as its specific co-chaperone⁴. Inactivation of yeast auxilin (Swa2) leads to anomalous accumulation of coated vesicles⁶, and RNA interference with nematode auxilin causes similar defects⁷. Mammalian genomes encode two auxilins: auxilin 1 is brain specific⁴, whereas auxilin 2 is general⁸. Auxilin 1 is a 910-residue protein with an amino-terminal PTEN-like domain, a central clathrin-binding domain, and a C-terminal Hsc70-binding J domain⁴. Auxilin 2 contains an additional N-terminal kinase domain⁹. Binding to clathrin coats, Hsc70 recruitment and *in vitro* uncoating require only residues 547–910, which comprise the clathrin-binding and J domains^{8,10}. The clathrin-binding region of auxilin appears to be largely unstructured in solution¹⁰, but about 25 residues at its C-terminal end contribute two additional helices to the usual three helices of the globular J domain, as revealed by a crystal structure¹¹ of a fragment comprising residues 810–910 and a solution structure¹² of a slightly larger fragment (residues 776–910). A recent image reconstruction of clathrin coats assembled with full-length auxilin instead of AP-2 adaptor proteins has shown that auxilin forms a shell of density within the coat¹³, but the resolution of the map does not allow specific visualization of clathrin–auxilin contacts.

We expressed and purified the 39-kDa C-terminal fragment of auxilin 1 (residues 547–910) comprising the clathrin-binding and J domains. Published studies show that this fragment can direct uncoating by interaction with Hsc70 (ref. 14). We used pre-assembled clathrin coats (containing three-legged clathrin heavy and light chain triskelions) and AP-2 complexes assembled as described in the accompanying paper⁵, and obtained site-specific binding of the auxilin fragment by adding increasing amounts of auxilin to coats (Fig. 1). Saturation corresponded to three auxilin molecules bound per clathrin triskelion, in agreement with previous

reports^{14,15}. To prepare electron cryomicroscopy samples, we mixed assembled coats with auxilin(547–910) at a molar ratio (clathrin to auxilin) of 1:20 and incubated the mixture for 30 min. To preserve saturation of auxilin-binding sites, we did not remove unbound auxilin(547–910) and carried out the electron microscopy in the presence of an auxilin background. We selected images of hexagonal barrel coats and computed a three-dimensional reconstruction (Fig. 2) at 12 Å resolution as described in Methods.

The map details of auxilin-bound coats resemble closely those of auxilin-free coats⁵, but the dimensions of the auxilin-bound D6 barrels (roughly 670 × 700 Å) are more isometric than those of the unmodified barrels (655 × 700 Å). Inaccuracy in the determination of electron-microscopic magnification cannot account for the observed effect, as it would produce isotropic differences rather than a difference in axial ratio. The change in shape of the barrel prevented us from computing a difference map directly. We therefore fitted clathrin leg segments into the map, obtained nine averaging transformations as in the accompanying paper⁵, and identified density in the averaged map not accounted for by the clathrin model (Fig. 3). In the accompanying paper we describe by the “invariant hub assembly” the tripod and three proximal segments radiating from a vertex, the distal segments (belonging to triskelions centred at nearest-neighbour vertices), and the triangle of ankles (belonging to triskelions centred at second-nearest-neighbour vertices) at the foot of the tripod. Although the proximal, distal and helical tripod parts of the hub assembly superpose well, the orientation of the ankle segment is shifted in the auxilin-bound form, becoming more parallel to the proximal and distal segments and moving the terminal domain radially outward. These local changes must produce the global distortion in axial ratio. There may also be other, more subtle adjustments in clathrin contacts that we are unable to detect at the current level of precision.

Auxilin(547–910) lies close to the site at which the ankle segments cross (Fig. 4), as might be expected from the change in ankle orientation that its binding generates. Density that we ascribe to a

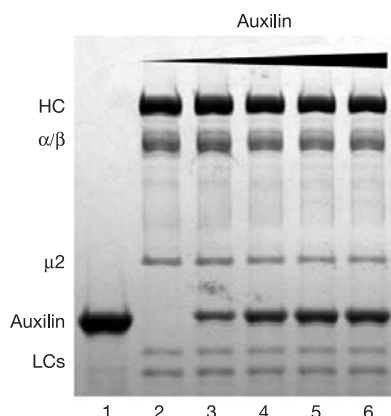


Figure 1 Binding of auxilin(547–910) to clathrin coats. The auxilin fragment was incubated with assembled coats at different molar ratios in reaction buffer for 30 min on ice. The coats were pelleted by high-speed centrifugation for 12 min at 60,000 r.p.m., 4 °C, in a TLA-100 rotor (Beckman). The protein composition of the re-suspended pellets was analysed by SDS-PAGE. Lane 1, auxilin(547–910). Lanes 2–6, pelleted coats after incubation with increasing amounts of auxilin(547–910) using initial molar ratios of auxilin to clathrin heavy chains (HC) as follows: 0:1 (lane 2), 1:1 (lane 3), 3:1 (lane 4), 10:1 (lane 5), 20:1 (lane 6). Saturation achieved by lane 5 corresponds to an estimated 1:1 molar ratio of auxilin(547–910) to clathrin heavy chain. LCs, clathrin light chains LCa and LCb; α/β and μ2, components of the AP-2 complex.

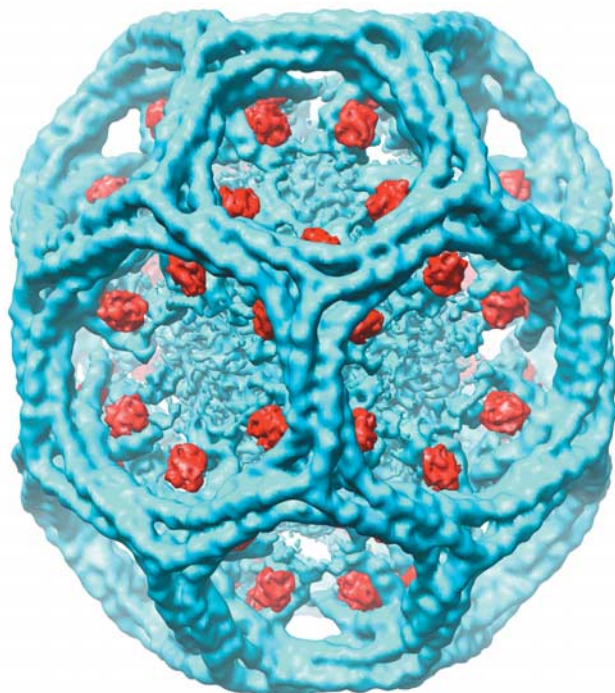


Figure 2 Three-dimensional image reconstruction of a clathrin D6 barrel with bound auxilin(547–910) at 12 Å resolution. The auxilin fragment (identified from local difference maps as described in the text) is rendered in red, whereas the rest of the structure is blue.

single auxilin(547–910) fragment merges (at the resolution of our map) with density from four different clathrin triskelions: a terminal domain, the two chains of the ankle crossing, and (probably) the C-terminal segment that extends from a tripod helix into the ankle-crossing zone (the ‘ankle brace’). The putative molecular contacts are consistent with *in vitro* pull-down experiments, showing that auxilin has multiple interactions with different segments of clathrin, including fragments that encompass the terminal domain and the ankle¹⁰.

The structure of the C-terminal 110 residues of bovine auxilin¹² was inserted by inspection into the difference density (Fig. 4). The fragment contains five α -helices, the last three of which are the canonical helices I–III of a J domain. The model of the five helices (residues 800–910) occupies about one-third of the volume that we attribute to the 39-kDa auxilin fragment. It does not seem to participate in any direct interaction with clathrin, in agreement with reported biochemical data showing that the J domain alone does not bind heavy chain⁴. The parts of the J domain believed to associate with Hsc70—the HPD triplet, helix II and the loop connecting helices I and II (ref. 11)—are all exposed on the outward-facing domain surface (Fig. 4c). The structure therefore shows that auxilin will direct Hsc70 to a position beneath the triskelion vertex, adjacent to the clathrin contact that we have proposed determines coat stability⁵.

The remaining domain(s) at the N terminus of auxilin, absent from our structure, would probably project inward towards the membrane. If the PTEN-like domain is indeed an active lipid phosphatase, then association of auxilin with a clathrin lattice will place it adjacent to potential substrates.

Proteins in the Hsp70 family, such as *Escherichia coli* DnaK, bind to and release short, exposed hydrophobic segments of polypeptide chain. ATP hydrolysis drives the cycle. The Hsp70 ATPase domain resembles actin; the substrate-binding domain is a molecular clamp with a peptide-binding groove. The coupling of the two domains

has yet to be visualized directly. A J-domain-containing protein recruits ATP-bound Hsp70 to the substrate (for example, an unfolded protein in the best-characterized examples); ATP hydrolysis and dissociation of the J protein leave the Hsp70 molecule clamped to an exposed hydrophobic segment; and binding of a new ATP opens the clamp and restarts the cycle. How can such a simple molecular device couple ATP hydrolysis to disassembly? The structures described here and in the accompanying paper⁵ lead to the following suggestions.

Clathrin light chains are not required for uncoating⁴. The best candidate for an unfolded, hydrophobic segment to which Hsc70 can attach is therefore the C terminus (residues 1631–1675) of the heavy chain, which projects into the corners of the triangular structure formed by three crossed ankle regions (Fig. 3a). This is the only part of the heavy chain that does not have a highly organized secondary and tertiary structure⁵. It contains the sequence QLMLT, which shares a noticeable resemblance to the last five residues of the FYQLALT peptide that binds Hsc70 with high affinity and efficiently stimulates its ATPase activity¹⁶. Moreover, the ankle crossing contacted by this part of the heavy chain is precisely the contact that is perturbed by auxilin binding. Depending on its relative orientation with respect to the ATPase domain, the Hsc70 clamp module could easily reach an exposed C-terminal peptide. The location of auxilin(547–910) and the changes in contacts among nearby clathrin leg segments thus suggest a mechanism by which auxilin could create local strain, release the neighbouring C-terminal segment from its interaction with an ankle, and recruit Hsc70 to clamp and sequester the segment thus exposed.

The change in overall geometry of the clathrin lattice generated by auxilin(547–910) binding also suggests a way in which a perturbation at one vertex could generate distortions at others. A large number of auxilin–Hsc70–C-terminal peptide interactions may not be required to destabilize a coat. If disassembly occurs in the absence of a pool of soluble clathrin that could replace dislocated trimers,

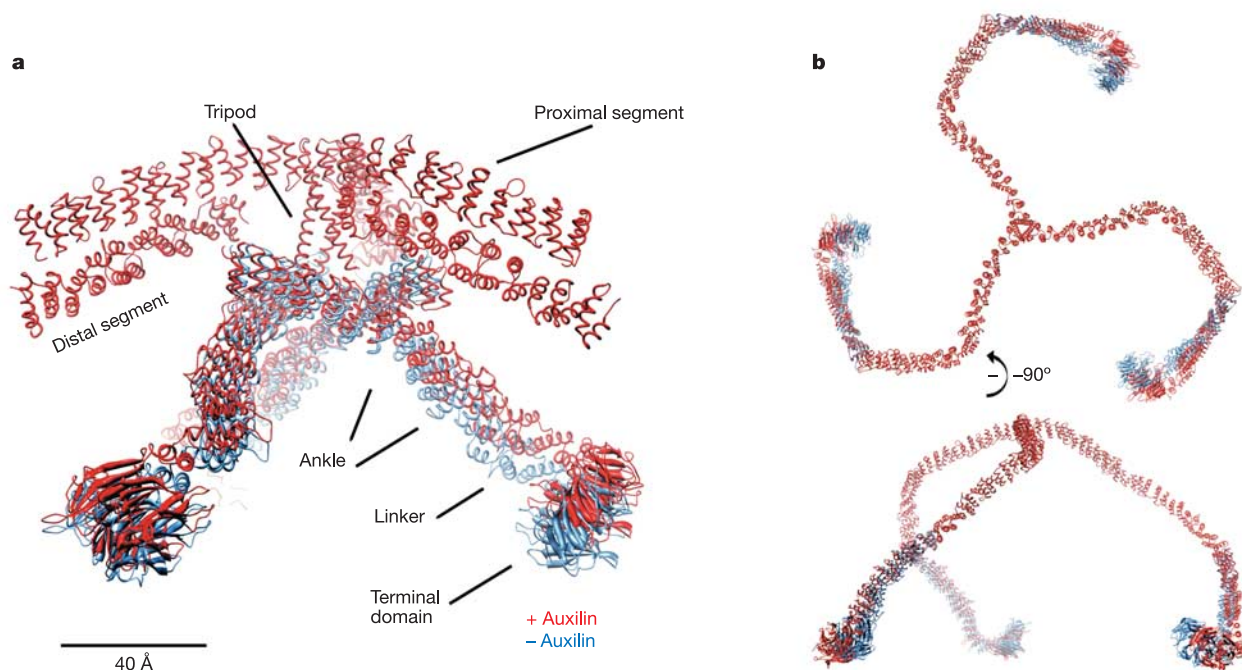


Figure 3 Changes in clathrin heavy-chain contacts produced by auxilin binding. **a**, Comparison of the arrangement of clathrin legs in the region around a vertex of the D6 barrel (the ‘hub assembly’, as defined in the accompanying paper⁵). The model from our 7.9 Å resolution analysis of a D6 barrel is in blue; the one obtained by adjusting that model

to fit the 12 Å auxilin-bound D6 barrel is in red. The ankle-crossing angles change in such a way that the terminal domains move radially outwards with respect to the outer shell. **b**, Superposition of clathrin triskelions from corresponding locations in the auxilin-bound D6 barrel (red) and the auxilin-free D6 barrel (blue).

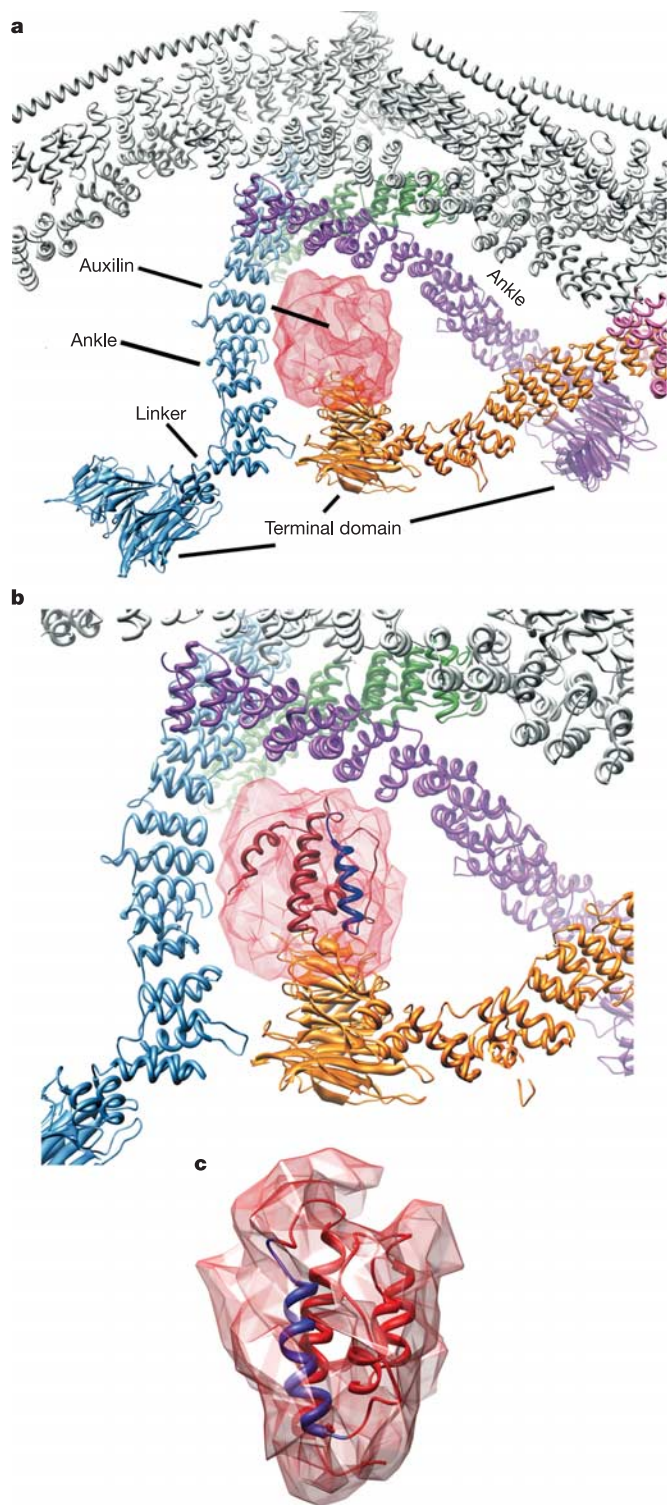


Figure 4 Fit of the auxilin J domain into the difference density. **a**, Auxilin difference density in relation to the hub assembly model. The view is the same as in Fig. 3a. A terminal-domain linker segment projecting towards this hub from another heavy chain centred three vertices away is in gold. The auxilin fragment fits into the intersection of the crossed ankles and also contacts the terminal domain emanating from a different hub. **b, c**, Different views showing the fit of the solution structure of the C-terminal, 110-residue fragment of bovine auxilin obtained by NMR¹² into the electron density map. Blue indicates the region of auxilin proposed to contact Hsc70 (ref. 11).

a few clamp-and-sequester events may be sufficient to initiate cooperative uncoating. □

Methods

Specimen preparation

Clathrin coats were assembled from purified clathrin triskelions and AP-2 complex proteins as described in the accompanying paper⁵. DNA encoding full-length bovine auxilin in a pQE-30 vector¹⁷ was provided by E. Ungewickell. The sequence encoding the clathrin-binding region and J domain (amino acid residues 547–910) was amplified from the full-length auxilin DNA by polymerase chain reaction (PCR) with Pfu DNA polymerase (Stratagene) using 5'-GACGTGAGATCTCCGAGTGGACCTACATCC-3' and 5'-CCTCCGCTCGAGTTAATACAAGGGCTTTTGGCCTTG-3' as the forward and reverse primers. The PCR product was digested with *Bgl*II/*Xho*I and subcloned into a *Bam*HI/*Xho*I-digested glutathione S-transferase (GST)-containing pGEX4T-1 vector (Pharmacia). The integrity of the subcloned auxilin DNA fragment was verified by sequencing.

The GST–auxilin(547–910) fusion protein was expressed in *E. coli* strain BL21. Bacteria were grown at 37 °C to an optical density at 600 nm of 0.6 and then induced with 1 mM IPTG at 25 °C for 3 h. The cells were spun down, re-suspended in 50 ml of PBS, pH 7.4, supplemented with one Complete Protease Inhibitor Cocktail tablet (Roche Applied Science), and lysed by sonication. Cell debris was separated by centrifugation and GST–auxilin(547–910) purified from the supernatant by affinity chromatography on glutathione Sepharose (Pharmacia) according to the manufacturer's instructions. The GST tag was removed by cleaving 1 mg of the Sepharose-bound fusion protein with 1 U of thrombin (Sigma-Aldrich) at 4 °C for 10 h. The cleavage reaction was stopped by addition of PMSF (1 mM final). GST-free auxilin(547–910) was further purified by gel filtration chromatography using a Superose 12 column (Amersham Biosciences) equilibrated with 20 mM HEPES, pH 7.0, 2 mM MgCl₂, 25 mM KCl, 10 mM (NH₄)₂SO₄. Purified auxilin(547–910) was stored in a frozen state at –80 °C.

Electron cryomicroscopy

To prepare samples for electron microscopy, assembled coats were mixed with auxilin(547–910) in reaction buffer at a molar ratio of 1:20 and incubated on ice for 30 min. To preserve saturation of all auxilin-binding sites, unbound auxilin was not removed. Freezing procedures and electron cryomicroscopy data collection were performed exactly as described in the accompanying paper⁵.

We recorded about 300 electron micrographs, which included the data collected from two sample batches prepared on different dates. Image processing was performed only for hexagonal barrel particles. About 6,500 particles were picked from the selected 190 high-quality micrographs and assembled into the stack. We did not go through the initial stages of particle image classification and class-average-based three-dimensional reconstruction for auxilin-bound particles. Instead, the three-dimensional model of auxilin-free hexagonal barrels (see the accompanying paper⁵) was used as a reference for direct parameter search and refinement in FREALIGN¹⁸.

We first performed FREALIGN refinement in the 800–40 Å resolution range to select the best particles. Images of auxilin-bound clathrin coats had relatively noisier backgrounds when compared with those of auxilin-free complexes mainly due to the presence of unbound auxilin. We therefore used a slightly less stringent criterion for particle selection and included about 900 clathrin coat particles with a PRES value lower than 65° (rather than 62°, as used for auxilin-free samples) in the final refinement procedure. Further refinement of the selected particles produced a 12 Å reconstruction of auxilin-bound clathrin coats as shown in Fig. 2.

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Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to T.K. (Kirchhausen@crystal.harvard.edu). Coordinates have been deposited in the Protein Data Bank under accession number 1XI5.

corrigenda

The Ras–MAPK pathway is important for olfaction in *Caenorhabditis elegans*

Takaaki Hirotsu, Satoshi Saeki, Masayuki Yamamoto & Yuichi Iino

Nature **404**, 289–293 (2000).

In this Letter, we used strain MT2124, the standard *let-60(n1046gf)* strain maintained in the *Caenorhabditis* Genetics Center, for odour-chemotaxis assays. However, we have found that this strain

carries a side mutation(s) that profoundly impairs chemotaxis to the odorant isoamyl alcohol, indicating that we need to re-evaluate our conclusion from the results shown in Fig. 1 that the *let-60(n1046gf)* mutant has a reduced efficiency of odorant chemotaxis. We outcrossed MT2124 to the wild-type N2 and obtained two *let-60(n1046gf)* strains, JN130 and JN131. We also outcrossed the MT4866 strain, the *let-60(n2021lf)* strain used in the study, and obtained the JN148 strain. All the outcrossed strains show reduced chemotaxis to the two odorants tested, isoamyl alcohol and diacetyl, at low odorant concentrations (T.H. and Y.I., unpublished results). The chemotaxis defects are comparable in extent to, or slightly weaker than, the original MT4866 *let-60(n2021lf)* strain. Our conclusion that both inactivation and hyperactivation of LET-60 Ras cause reduced chemotaxis therefore remains unchanged. However, the result shown in Fig. 1d, which suggested that *ksr-1(lf)*, *mek-2(lf)* and *mpk-1(lf)* suppress *let-60(n1046gf)*, is no longer valid because outcrossed *let-60(n1046gf)* strains do not show chemotaxis defects at the odorant concentration used in Fig. 1d (1×10^{-3} dilution of isoamyl alcohol). □

Contrasting origins of the upper mantle revealed by hafnium and lead isotopes from the Southeast Indian Ridge

Barry B. Hanan, Janne Blichert-Toft, Douglas G. Pyle & David M. Christie

Nature **432**, 91–94 (2004).

In this Letter, the quantity ε_{Hf} in Fig. 3 and its legend should read $\Delta\varepsilon_{\text{Hf}}$, which is the change in hafnium isotopic composition relative to the $\varepsilon_{\text{Nd}} - \varepsilon_{\text{Hf}}$ mantle array. □

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A dose of reality

Over the past few years, television on both sides of the Atlantic has been dominated by 'reality' programmes. People can't seem to get enough of watching ordinary people go about their daily routine or of seeing celebrities being thrust into frequently humiliating situations.

So the huge response over the past year for the four students who wrote their Graduate Journals for *Naturejobs* shouldn't have been a surprise. Thousands of online readers followed our correspondents' plights as they mulled over where to pursue a postdoc, pondered academia versus industry or wondered whether to leave the bench behind.

But there are key differences between reality shows and the *Naturejobs* column, the most significant of which is the level of reality. On TV it doesn't matter how 'real' the contestants are, they are still thrust into contrived situations. The students, by contrast, reported their ups and downs pretty much as they happened.

One of the more gratifying differences was what drove the interest in the students. On TV, it's often a matter of antipathy for the badly behaved contestants, hoping that they'll get some sort of comeuppance. For the Graduate Journals, interest was driven more by empathy. The students often received encouraging e-mails when they reported setbacks such as failed experiments. That reaction underscores the reason for publishing the journals: to show graduate students that others, too, face adversity.

And although our students might sometimes have felt that they were in a show called "I'm A Graduate Student, Get Me Out of Here", in the end, a more appropriate title would have been "Survivor", as you'll see from each student's final entry, made available online over the next few weeks (www.nature.com/naturejobs/channels/graduate/graduate-journal.html).

Paul Smaglik
Naturejobs editor



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